THE ROLE OF RETROGRADE REPRESSION IN LIMITING AXONAL REGENERATION IN THE CENTRAL NERVOUS SYSTEM

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ABSTRACT

The regenerative capacity of mature mammalian CNS neurons after axonal injury is severely limited by a variety of mechanisms. Retrograde repression is the continuous inhibition of the expression of growth phenotypes by tonic signals produced by target tissues and transmitted to the neuron cell body via retrograde axonal transport. Loss of target contact through axonal injury is thought to interrupt this retrograde signal and allow the up-regulation of growth-associated proteins. Most CNS neurons, however, possess many widespread axon collaterals, such that retrograde repression is maintained by intact sustaining collaterals even if some axons are injured.

In this project we investigated whether or not retrograde repression plays a role in limiting the expression of GAP-43 in transcallosal neurons. Because TCNs possess local axon collaterals to nearby cortex and project distal axons to homologous areas of contralateral cortex, we hypothesized that the simultaneous interruption of retrograde repressive signals from both ipsilateral and contralateral cortex would result in an up-regulation of GAP-43 expression in at least some TCNs.

We found that a bilateral infusion of a function blocking antibody to FGF-2 into the parietal cortex of rats using implanted osmotic mini-pumps resulted in a significant increase in the level of expression of GAP-43 mRNA in TCNs identified by retrograde fluorescent labeling. In contrast, neither ipsilateral or contralateral antibody infusions alone increased GAP-43 expression significantly compared to controls. The level of expression of GAP-43 in TCNs did not significantly increase after stereotactic callosotomy alone, but callosotomized animals treated with an ipsilateral infusion of anti-FGF-2 had levels of increased GAP-43 expression equivalent to those seen in animals that had received bilateral antibody infusions.

We conclude that FGF-2 provides a retrograde repressive signal for at least some mature mammalian TCNs, and that the expression of growth-associated proteins can be up-regulated in CNS neurons by simultaneously blocking retrograde repressive signals from all existing axon collaterals. The ability to alter the gene expression of mature CNS neurons in both normal and injured states through the targeted infusion of a pharmacological agent may have potential clinical implications in the future.

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PNS – peripheral nervous system
CNS – central nervous system
GAP – growth associated protein
DRG – dorsal root ganglion
TCN – transcallosal neuron
FG – fluorogold™

BACKGROUND

1.0 INTRODUCTION

The human central nervous system is arguably the most complex entity in the natural world. Several kilograms of neurons and associated glia, projecting elaborate arborizations of axons and dendrites to form endlessly interconnected circuits, from which emerges coordinated movement, memory, language, emotion, symbolic thought, and consciousness. This system, however, possesses one great weakness, for its regenerative capacity is severely constrained. After maturity, only a tiny fraction of the neurons destroyed by traumatic or degenerative processes and other environmental insults can be replaced by the production of new cells. Damaged axons are unable to regenerate. Disrupted connections can rarely be restored.

This limitation of regeneration has profound implications for the outcome and prognosis of traumatic and metabolic injuries to the brain and spinal cord. Clinicians have long known that the prospect for recovery after injuries to the central nervous system is almost universally grim. Plastic rearrangement of undamaged circuitry to restore lost functions can occur, but only to a very limited degree. The cost to society of brain and spinal cord injury, which frequently affect young individuals in the prime of their productive years, is incalculable. Thus there is much interest in neuroscience research to elucidate the mechanisms that regulate the growth and regeneration of axons, and ultimately develop therapeutic interventions than can promote axon regeneration after brain and spinal cord injury in such a way that allows for the recovery of lost functionality.

1.1 RESPONSES TO AXONAL INJURY: AN OVERVIEW OF NORMAL AXONAL REGENERATION

Successful regeneration after axonal injury requires a sequence of interrelated events. First, the injured neurons have to survive the acute phase of the injury. Second, they must activate the necessary genetic programs to produce a phenotype capable of regeneration. Third, the regenerating axons have to navigate long distances back to the appropriate target tissues. Finally, once there they then have to reconstitute functional synaptic connections. Only if all these steps are satisfactorily completed will the injured neurons be able to regain their lost functionality.

Virtually all neuron types with axons projecting out into the peripheral nervous system (PNS) display a typical metabolic response and are capable of undergoing lengthy regenerative growth following axotomy. While the distal axon stump commences a process of active degeneration known as Wallerian degeneration, and inflammatory cells infiltrate the injury site, the neuron cell body undergoes a sequence of changes, known as chromatolysis, whereby the cell body swells in size, the nucleus moves to an eccentric position, and the nissl substance moves to the periphery of the cytoplasm. Local presynaptic processes may detach from the neuron cell body and its dendritic tree. After a few days, rates of mRNA and protein synthesis increase, marking the activation of genetic programs for the regenerative phenotype. (Grafstein, 1975) At this point, injured neurons may initiate axonal regeneration, enter a stable quiescent state, or progress to apoptotic cell death. Target-derived trophic factors appear to play a role in neuron survival. (Grafstein, 1975; Hall, 2005) More proximal axotomies tend to be more likely to result in neuronal death, and neurons possessing intact axon collaterals are also more likely to survive. Axon sprouts can appear at the proximal stump within hours of axotomy, and if the regenerative phenotype is successfully initiated, axon elongation will initiate in days. (Hall, 2005)

In peripheral nerves, regenerating axons follow the now emptied fascicles of the distal nerve stump back to the target tissue, provided the nerve itself remains intact, or has been surgically repaired. Pathfinding failure may result in non-functional aberrant reinnervation, or painful neuromas.

In contrast to PNS neurons with their robust regenerative capability, mammalian central nervous system (CNS) neurons almost always fail to regenerate injured axons. Some CNS neurons progress to cell death or atrophy, while many others exhibit no response of any kind, with no evidence of chromatolysis.(Grafstein, 1975) Even if regeneration initiates at the axon stump, regrowth beyond the injury site almost never occurs.(Skene, 1984; Yiu and He, 2006)

Why should the regenerative capacity of CNS neurons be so limited compared to neurons of the PNS? An individual neuron's ability to regenerate axons appears to be dependent on a wide range of intrinsic and extrinsic factors. Clearly, different types of neurons possess differing capacities for axon regeneration. For example, embryonic or developing neurons are more capable of regeneration than mature neurons, and the CNS neurons of anamniotes (fish and amphibians) are more capable of regeneration than the CNS neurons of amniotes (reptiles, birds, and mammals).(Skene, 1984)

Extrinsic factors in the neuronal microenvironment also play an important role in determining the capacity of a neuron to regenerate, and can account for some of the observed differences in the regenerative abilities of PNS versus CNS neurons. Unlike CNS neurons, whose axons all terminate exclusively within the CNS itself, PNS neurons possess axons that terminate in target tissues both inside and outside the CNS, and are thus exposed to regulatory factors external to the CNS. One crucial difference is the nature of the glial cells associated with these two types of neurons. In peripheral nerves, denervated Schwann cells rearrange into structures called Bungner bands that provide a guiding scaffold for regenerating axon sprouts, (Skene, 1984) and produce substances that help to create a temporarily permissive microenvironment for axon regeneration, (Hall, 2005) which is usually maintained until successful target re-innervation is achieved.(Grafstein, 1975)

In contrast, CNS glial cells (oligodendrocytes and astrocytes), do not produce a locally permissive environment for regeneration in response to injury, the way Schwann cells in the PNS do, (Qiu et al., 2000; Yiu and He, 2006) and in fact tend to be inhibitory of axon growth. In addition, the inflammatory reaction to axon injury in the CNS has been shown to generate yet more inhibitory factors that act to prevent axon regeneration.

(Bandtlow and Schwab, 2000; Rhodes et al., 2003; Yiu and He, 2006; Zurn and Bandtlow, 2006)

The final factor affecting successful axon regeneration is time. There is a limited window of opportunity during which axon regeneration needs to be completed. Neurons cannot sustain regenerative phenotypes indefinitely. Even in the PNS, chronically denervated Schwann cells will eventually apoptose,(Hall, 2005) and chronically denervated target tissue may eventually lose the ability to accept new synapses. Thus the longer it takes for axon regeneration to be completed, which is affected by factors such as the distance the regenerating axons must travel and the ease of navigating to the appropriate target tissue, the less the likelihood of a favorable outcome.

1.2 GROWTH ASSOCIATED PROTEINS AND THE GAP HYPOTHESIS

Experiments conducted in the early 1980s found that in neurons capable of axon regeneration, certain proteins normally not present or present in low concentrations, became expressed at high levels in response to axotomy.(Skene and Willard, 1981b) Neuron populations that were not capable of regenerating their axons after injury did not increase their expression of these same proteins in response to axotomy.(Skene and Willard, 1981a; Skene, 1984; Reh et al., 1987) Further experiments found that these same proteins were expressed at high levels in development during periods of axon outgrowth in a variety of neuronal populations.(Skene and Willard, 1981a; Kalil and Skene, 1986; Skene, 1989) Thus it appeared that axonal regeneration and developmental axon growth were related phenomena, dependent on the same set of genes, and that regeneration may be considered in many senses as a recapitulation of developmental growth.

These proteins became known collectively as growth-associated proteins (GAPs). Some of the first GAPs to be characterized were membrane bound proteins that were rapidly transported to growing axon terminals.(Skene and Willard, 1981a) The observed timing of the up-regulation and down-regulation of various GAPs also coincided with the various observed stages of axon elongation, branching, and synapse formation.(Skene, 1989) These and other experimental findings lead to the development of the GAP hypothesis. Namely, that GAPs play essential roles in axon outgrowth and regeneration,

and their expression is required for successful axonal growth or regeneration. GAP expression creates the growth phenotype used by all neurons during development of their axon arbors, and neurons regenerate injured axons by reactivating, in part or in whole, this developmental growth phenotype. Those neurons which are incapable of axon regeneration, such as adult mammalian CNS neurons, are neurons which either cannot or are somehow prevented from up-regulating GAP expression in response to axonal injury. In order to understand the process of axon regeneration, it is therefore necessary to understand how axons grow during development.

1.3 AN OVERVIEW OF DEVELOPMENTAL AXON GROWTH AND PATHFINDING

1.3.1 The Initiation of Axon Outgrowth and the Establishment of Neuronal Polarity

During development, most CNS neurons are born in ventricular or paraventricular proliferative zones, and then migrate sometimes substantial distances to their final destinations, following a variety of physical and chemical cues. For example, pyramidal projection neurons are generated in the ventricular zone and migrate outwards to assemble the six layers of the mammalian neocortex in an inside to outside fashion, such that deeper layers are comprised of the oldest cells. In contrast, nonpyramidal neurons that will perform inhibitory functions in cerebral cortex are generated in the paraventricular ganglionic eminences and migrate tangentially to their final locations.(Bielas et al., 2004)

Neurons begin differentiating soon after completing migration, and start to elaborate the many cellular processes that will become dendrites and axons in maturity. The typical CNS neuron possesses a single axon which may or may not have branches, and many dendrites arising from the main cell body.(Dotti and Banker, 1987) The establishment of this polarity is one of the first steps in axon growth. In vitro experiments with hippocampal(Dotti et al., 1988) and cortical(Kalil et al., 2000) neurons reveal that at least for these two neuronal subpopulations in cell culture, the mature polarized form is acquired via a stereotyped sequence of developmental events. Multiple minor processes start to grow out from the neuron cell body, and are at first

indistinguishable from one another. At some point, one of the minor processes becomes selected for an axonal fate, and its growth rate is accelerated while the growth of the remaining minor processes is suppressed. After this process differentiates into the axon, the remaining minor processes begin growing again, and eventually become dendrites.(Dotti et al., 1988)

1.3.2 Axon Pathfinding During Development

The axons of CNS projection neurons often must traverse long and frequently convoluted paths to reach distant innervation targets, with multiple bends and sharp turns. The developmental mechanisms by which these axons find their way to their targets remain an area of considerable research.

Axon elongation and pathfinding is a function of the growth cone, a motile structure at the tip of the growing axon. Morphologically, the growth cone is a cytoplasmic swelling possessing multiple lamellipodia. As the growth cone migrates, it lays down the axon behind it along the path it travels. The course of the mature axon thus reflects the path that the growth cone navigated in development from the neuron cell body to the target tissue.

The growth cone's morphology has been observed to increase in complexity whenever and wherever a growing axon is required to make important directional decisions.(Dingwell et al., 2000) Morphologically complex lamellipodia extending in different directions appear to compete with one another until a new direction becomes selected and the lamellipodia heading in other directions are retracted. As the growth cone flows in the selected direction, the microtubule array in its base is reoriented in the new direction and subsequently is assembled into a new segment of axon as the growth cone passes. This process continuously repeats itself to lay down further segments of axon until the whole process is halted when the axons approach appropriate target tissues.(Brandt, 1998)

The available evidence suggests that growth cones make navigational decisions based on various cues they sense in the microenvironments they traverse. Mechanisms implicated in growth cone guidance include adherence to preferred physical substrates, and attraction to or repulsion by chemotactic substances, both membrane-bound and in

soluble diffusion gradients. Several families of molecules have been identified that have effects on growth cone navigation and the growth cones' responses to these environmental cues can also be variable and time dependent. The growth cone of one axon can be attracted to a substance during one period of growth and repelled during another, or become sensitive to a certain substance only during specific periods of time.(Brandt, 1998; Dingwell et al., 2000; Cooper, 2002) Changes in responsiveness to various substances can be mediated by yet other environmental cues, or may constitute part of an intrinsic program in the neuron itself. In some axon tracts, the first pioneering fibers to reach a target site become an additional guidance mechanism in their own right, forming a physical substrate that subsequent axons can follow through cell adhesion to the same target site. (Richards, 2002)

The axons of different populations of neurons use different combinations of these mechanisms to reach their target sites. The guidance of axons to their target sites is an intricate process requiring precise timing and coordination of multiple developmental processes in the neurons themselves, the target site, and in all the tissues traversed by the axons during their growth. The exact sequence of events is specific for each individual neuronal subpopulation, and the majority of these sequences remain unknown.

1.3.3 The Development of Axon Collateral Branches

Neurons may elaborate axon arbors with multiple collateral branches to different distant target sites. Collateral branching also plays an important role in target recognition in many populations of cortical neurons. In these neurons, axons are frequently observed to overshoot their ultimate targets, only to later grow collateral branches into the target, followed by retraction of the overshot portion of the axon, and further selective refinement of the axon arborization.(Kalil et al., 2000)

Early observations suggested that axon collaterals do not form, as one might at first expect, from primary bifurcations of migrating growth cones as is the case in the elaboration of terminal arborizations within a target area.(O'Leary and Terashima, 1988) Rather, collateral branches appeared to arise from the main axon shaft after the main growth cone had already passed and advanced a substantial distance beyond. More detailed observations in both *in vivo* and *in vitro* experiments demonstrated that the

growth cone actually pauses for significant amounts of time at the sites of future collateral branching. During this period of pausing, the growth cone enlarges, and when forward migration resumes, structural elements of the growth cone are left behind, out of which the collateral branch later grows, with its own growth cone.(Kalil et al., 2000)

Thus it now appears that the formation of axon collaterals does, after all, involve what is essentially the bifurcation of the primary growth cone, except that it occurs in a delayed fashion. The pause in the progress of the primary growth cone and its subsequent expansion in size appear to allow for the accumulation and rearrangement of cytoskeletal elements necessary in creating the new collateral branch and secondary growth cone associated with it.

Collateral branching appears to be mediated by target derived factors. Chemorepulsive factors have been implicated in causing the primary growth cones to pause, while chemoattractive gradients play important roles in initiating the growth of collateral branches and guiding them to their targets. The same groups of substances implicated in primary axon guidance also appear to be involved in the creation of collateral branches.

1.3.4 The Role of GAPs in Axon Outgrowth and Collateral Branching

In support of the GAP hypothesis, many of the proteins found to play important roles in the assembly and construction of the axon shaft and growth cone, and in mediating the sensitivity of the growth cone to guidance cues were subsequently identified as GAPs. That is, they displayed the expression pattern typical of GAPs, in which they are expressed at high levels during development and regenerative phases, but are down-regulated in mature, functioning neurons. These proteins included cytoskeletal proteins used to assemble the axon shaft and growth cone, proteins involved in lamellipodia motility, receptors and other intermediates in signal transduction systems associated with guidance cue molecules, and proteins necessary in mediating changes in growth cone morphology.(Skene, 1984, 1989; Meiri and Gordon-Weeks, 1990; Jenkins and Hunt, 1991; Brandt, 1998; Dingwell et al., 2000; Kalil et al., 2000; Andrews et al., 2006)

It became clear that the high regenerative capacity of developing neurons was due to their high levels of GAP expression, and that the growth phenotype is subsequently turned off as developing neurons mature. Neurons with injured axons had to reactivate their developmental growth phenotypes in order to regain the potential for axon regeneration. This explained why neurons that failed to up-regulate GAP expression after axon injury also failed to regenerate the injured axons.

1.4 THE ROLE OF GAP-43 IN AXON GROWTH AND REGENERATION

1.4.1 Correlative Observations

The protein GAP-43, also known variously as pp46, B50, F1, and neuromodulin, was one of the first discovered and best characterized of the growth associated proteins.(Meiri et al., 1986; Benowitz and Routtenberg, 1997) Not long after GAP-43 was first associated with axonal growth and regeneration,(Skene and Willard, 1981a) it was determined that the protein was very similar in its properties to proteins that had been previously identified, characterized, and named in a variety of other contexts. It was eventually determined by sequencing that all these proteins were, in fact, the same.(Benowitz and Routtenberg, 1997) After the initial identification of GAP-43 in frogs, homologues were discovered in many different vertebrates spanning the evolutionary spectrum, including fish, rodents (mice, rats, hamsters), rabbit, cow,(Skene, 1989) chicken,(Moss et al., 1990) and primates.(Benowitz and Routtenberg, 1997)

During development, GAP-43 was found to be expressed in high levels in virtually all neuronal subpopulations in the CNS and PNS. Some of the places and neuronal populations where GAP-43 activity has been studied include retinal ganglion cells,(Skene and Willard, 1981a; Reh et al., 1987; Doster et al., 1991; Benowitz et al., 1998; Sretavan and Kruger, 1998; Soto et al., 2003) corticospinal tract,(Reh et al., 1987; Karimi-Abdolrezaee and Schreyer, 2002; Karimi-Abdolrezaee et al., 2002) rubrospinal tract,(Tetzlaff et al., 1991; Fernandes et al., 1999) hippocampal neurons,(Goslin et al., 1988; Goslin and Banker, 1990; Goslin et al., 1990; Aigner et al., 1995; Shen et al., 2002) transcallosal neurons,(Shen et al., 2002) purkinje cells,(Dusart et al., 2005) facial motor neurons,(Tetzlaff et al., 1991) spinal cord motor neurons,(Bisby et al., 1996; Fernandes et

al., 1999) and dorsal root ganglion (DRG).(Bisby, 1988; Schreyer and Skene, 1993; Liabotis and Schreyer, 1995) GAP-43 is substantially down-regulated in most adult CNS neuron populations. Mature neurons that continue to express detectable levels of GAP-43 include neurons in the thalamus, basal ganglia, hippocampus, and sensory association cortex.(Skene, 1989) Many of these are areas of the central nervous system that possess a high degree of plasticity in their synaptic connections throughout adult life, such as areas of higher level sensory association cortex and limbic and hippocampal areas associated with memory. However, not all neuron populations known to remain highly plastic express elevated levels of GAP-43 in maturity, and not all the areas where GAP-43 levels are known to remain elevated in the adult are known to possess high degrees of plasticity.

The beginning of GAP-43 up-regulation in development coincides with the initial outgrowth of the axon and in many neurons GAP-43 levels remain elevated up to the time of synaptic maturation.(Skene, 1989) The down-regulation of GAP-43 expression appears to occur in two distinct phases, with the first decrease occurring at the time when growing axons first begin to contact their target tissue, and a second decrease at the time of the maturation of the terminal arborization. (Karimi-Abdolrezaee et al., 2002)

In mature neurons capable of axon regeneration, GAP-43 has been shown to be up-regulated in response to axotomy to levels equivalent to those seen during development.(Skene and Willard, 1981b; Bisby, 1988; Tetzlaff et al., 1991; Fernandes et al., 1999; Soto et al., 2003) In keeping with the GAP hypothesis, mammalian CNS neuron are mostly not able to regenerate their axons and do not show elevation of GAP-43 expression after axotomy.(Kalil and Skene, 1986; Reh et al., 1987) The reliable association observed between elevated expression of GAP-43 and axonal growth and regeneration has lead to the use of GAP-43 as an experimental marker for the neuronal axon growth and regeneration phenotype, regardless of the actual function of the GAP-43 protein.

Later discoveries found that there were some mammalian CNS neurons capable of up-regulating GAP-43 expression after axotomy. These included retinal ganglion cells(Doster et al., 1991) and the neurons of the rubrospinal tract, (Tetzlaff et al., 1991; Fernandes et al., 1999) Most of these neurons however, still did not normally regenerate

their axons, unless a permissive environment was provided, for example by implanting a peripheral nerve graft. Similar findings were observed in experiments with dorsal root ganglion (DRG) cells. Injured central axons of DRG cells could regenerate into a peripheral nerve graft when GAP-43 expression in the DRG cell body was highly up-regulated in response to simultaneous injury of the peripheral axon, but not otherwise. (Richardson and Issa, 1984) Thus, GAP-expression is associated with the ability to regenerate axons, but successful regeneration could be thwarted by other environmental factors.

The distribution of GAP-43 protein within the neuron was also highly suggestive of a role in growth and regeneration. GAP-43 was found to be rapidly compartmentalized into axons almost immediately after the establishment of neuronal polarity and an axon could be morphologically identified. GAP-43 levels in the other minor processes fell rapidly at this time and GAP-43 became virtually undetectable in the dendrites that subsequently developed.(Goslin et al., 1988; Goslin and Banker, 1990; Goslin et al., 1990) Within the axon, GAP-43 was concentrated in the growth cone.(Meiri et al., 1986) More detailed investigations found that within the growth cone, GAP-43 was tightly associated with the cell membrane, and more specifically, with the membrane skeleton, the network of proteins that supports and stabilizes the cell membrane in the growth cone and allow for changes in shape.(Meiri and Gordon-Weeks, 1990; Moss et al., 1990; Strittmatter et al., 1992) Furthermore, GAP-43 was most highly concentrated in the areas of growth cone membrane that were most tightly adherent to physical substrates.(Meiri and Gordon-Weeks, 1990) Taken together, the localization of GAP-43 protein in the growth cone suggested some role in substrate adhesion and recognition and the morphological changes required for growth cone motility.

1.4.2 Biochemical and Structural Properties

The gene for GAP-43 is found on chromosome 3 in humans and chromosome 16 in mice. The first 57 amino acids from the N-terminus are highly conserved in vertebrates. Despite its strong association with the membrane skeleton, GAP-43 does not possess a true transmembrane domain. The membrane binding region is comprised of the first ten amino acids of the N-terminus. Fatty acid chains are known to post-

translationally modify cysteine residues in the amino terminal, although the role of such binding in membrane association remains unclear.(Benowitz and Routtenberg, 1997)

GAP-43 is phosphorylated by protein kinase C. Phosphorylation of GAP-43 has been associated with long term potentiation,(Skene, 1989; Benowitz and Routtenberg, 1997) axon sprouting,(Aigner et al., 1995) and the stimulation of neurite outgrowth by cell surface adhesion.(Meiri et al., 1998)

Except for the small membrane binding domain, the GAP-43 protein is highly hydrophilic, with an acidic isoelectric point of 4.3 to 4.5. It has an unmodified molecular weight of 23.6 kD. Structurally, GAP-43 extends away from the inner surface of the cell membrane and into the cytoplasm, allowing for interactions with cytoskeletal and soluble cytoplasmic constituents. It has also been proposed that GAP-43 might be capable of reversibly detaching from the membrane and becoming a soluble protein in the cytoplasm.(Skene, 1989)

GAP-43 plays a role in a variety of cellular second messenger systems. It interacts with G proteins, and may alter their responsiveness to stimulation by various receptors. (Strittmatter et al., 1992) It is also involved in the phosphatidylinositol second messenger system. Unphosphorylated GAP-43 binds calmodulin preferentially in the absence of calcium.(Alexander et al., 1987; Benowitz and Routtenberg, 1997)

1.4.3 Genetic Knock-out Studies

When GAP-43 expression was inhibited in chick neurons using antisense mRNA, the neurons were observed to be able to extend only thin axons with atrophic growth cones, which displayed profound defects in their ability to interact with extrinsic guidance cues.(Benowitz and Routtenberg, 1997) Transgenic experiments have lent further support to the notion that GAP-43 plays a role in axon pathfinding. Homozygous nonfunctional GAP-43 mouse mutants do not survive long after birth. In one study, the brains of mutant embryos appeared grossly normal, but microscopically had multiple aberrations of axon pathfinding in the optic chiasm of their midline crossing fibers, where these fibers seemed to wander randomly back and forth across the midline before exiting the chiasm.(Sretavan and Kruger, 1998)

In another experiment, homozygous mutants failed to form several major commissural tracts, including the anterior commissure, the corpus callosum, and the hippocampal commissure. (Shen et al., 2002) Heterozygous mutants possessing one wild-type GAP-43 allele displayed variable phenotypes, with the severity of malformations correlating to individual levels of GAP-43 phosphorylation. GAP-43 deficient neurons displayed no abnormality in axon elongation compared with wild-type neurons, but instead had marked deficiencies in the ability of their axons to fasciculate with each other.

The impairment of axon fasciculation again suggests a role for GAP-43 in the recognition of, binding to, and/or response to physical substrates. Other experiments lend further support to this possibility. Cerebellar neurons deficient in GAP-43 were found to be unable to extend their axons in response to stimulation by the cellular adhesion molecule NCAM, although they retained the ability to extend axons in response to other known trophic factors.(Meiri et al., 1998)

1.5 THE RETROGRADE REPRESSION OF GAP EXPRESSION

1.5.1 GAP-43 Expression is inhibited by target-derived factors

As previously stated, the expression of GAP-43 begins to decline at the time when target contact starts to be established. This observation has lead to the speculation that the expression of GAP-43 might be regulated by target-derived factors, and lead to the development of the concept of retrograde repression as one mechanism accounting for the down-regulation of GAP expression in mature neurons. This is illustrated in figures 1.1 and 1.2. Retrograde repression is the continuous inhibition of GAP expression by target-derived signals that are transmitted to the neuron cell body from the target tissue along the axon by retrograde axonal transport. Axonal injury in responsive neurons interrupts this inhibitory signal, which enables the up-regulation of GAP-43 and other growth associated proteins, and allows for the initiation of the regenerative phenotype.

Experiments have lent support to the theory that retrograde repression plays an important role in the regulation of GAP expression in PNS neurons. When the axons of

dorsal root ganglion cells were disrupted by a crush axotomy of the sciatic nerve, substantial up-regulation of GAP-43 expression was seen in response, followed by down-regulation back to baseline levels around the time when target contact was re-established.

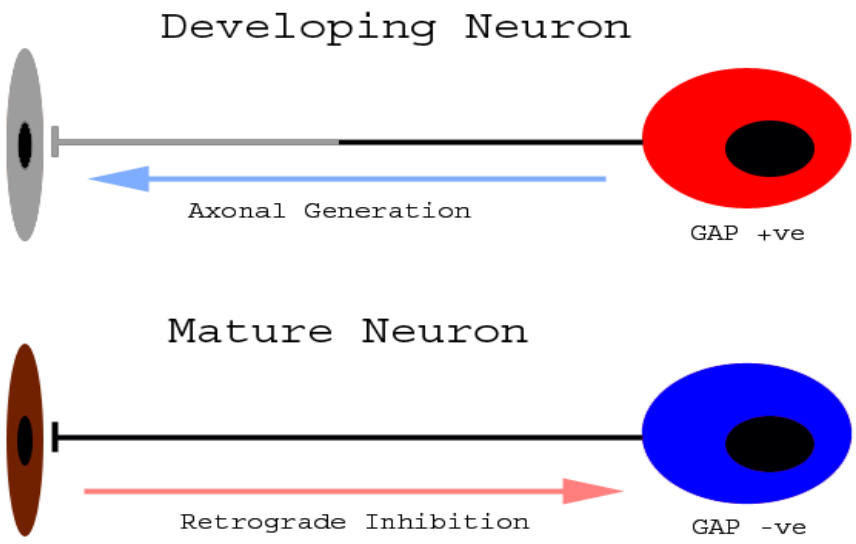

Figure 1.1: Retrograde repression of GAP expression during development. Arrival at the target site brings the developing axon into contact with target derived factors that suppress GAP expression and further axon growth. In mature neurons, a tonic inhibition is maintained by continued target contact.

When the axons were disrupted by a segmental resection of the sciatic nerve that prevented successful reinnervation, GAP-43 expression remained high and was not down-regulated at the expected time.(Bisby, 1988) Other experiments found that the magnitude of the induction of GAP-43 following axotomy in DRG neurons did not depend on the distance of the axotomy from the neuron cell body, suggesting that the repressive factor was produced at the target site, rather than somewhere along the course of the peripheral nerve.(Liabotis and Schreyer, 1995) Further experiments with motor neurons showed that the retrograde repressive signal was dependent only on target contact and not target activity.(Bisby et al., 1996)

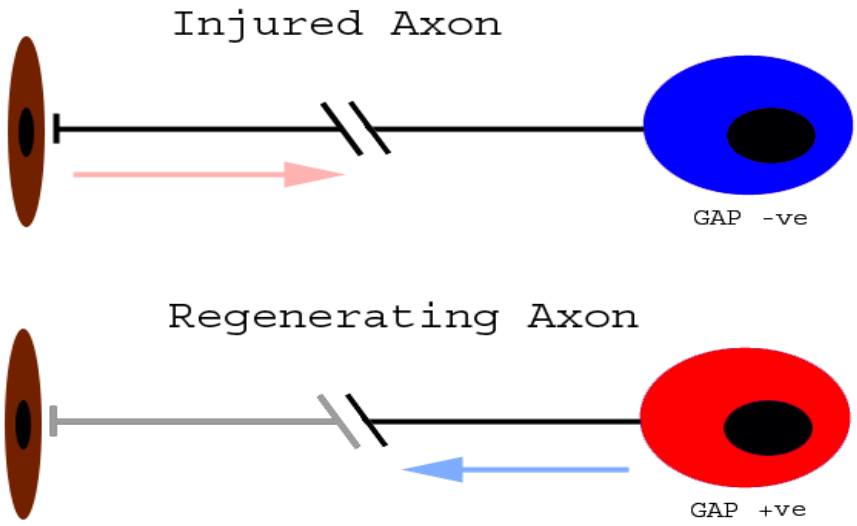

Figure 1.2: The interruption of retrograde repression by axonal injury. The loss of tonic inhibition allows for up-regulation of GAP expression and the initiation of axonal regeneration.

Just as in PNS neurons, the down-regulation of GAP-43 expression during development in CNS neurons coincides with the beginning of target contact.(Karimi-Abdolrezaee et al., 2002) A delay in the down-regulation of GAP-43 can be observed in some corticospinal tract neuron subpopulations if retrograde axonal transport is blocked by colchicine during this time period. When corticospinal tract neurons at an earlier stage of development were exposed *in vivo* to an infusion of a target tissue extract obtained from adult spinal cord, a premature down-regulation of GAP-43 expression was observed, indicating that a factor present in the adult target tissue does indeed act to inhibit GAP expression, and that retrograde axonal transport is required to carry this signal to the neuron cell body.(Karimi-Abdolrezaee and Schreyer, 2002)

1.5.2 Retrograde Repression May be Maintained in the CNS by Sustaining Collaterals

Dorsal root ganglion (DRG) cells are useful models for studying retrograde repression because they possess axons that divide near the cell body into two branches, one within the PNS innervating peripheral targets and the other in the CNS projecting back into the spinal cord. Transection of the peripheral branch results in a robust up-regulation of GAP-43, but transection of the central branch does not.(Schreyer and Skene, 1993)

If both axon branches of a DRG neuron are cut, GAP-43 is up-regulated and the peripheral axon is regenerated. The central axon usually does not successfully regenerate, but if it is exposed to the favorable environment of a peripheral nerve graft, it will grow into the graft. (Richardson and Issa, 1984) Indeed, even if the central axon is not injured, an injury to the peripheral axon can induce the extension of central axons into a peripheral nerve graft. (Richardson and Verge, 1986)

These experimental findings can be explained by postulating that the peripheral axon of the DRG acts as a sustaining collateral that carries retrograde repressive signals while the central axon does not. Thus injury to the central axon alone cannot induce GAP-43 up-regulation because the repressive signal continues to be provided by the peripheral axon, while injury to the peripheral axon eliminates the retrograde repression, allowing the DRG to activate a growth phenotype that can promote the growth of new central axons, or possibly even the extension of pre-existing and previously undamaged central axons, if a permissive environment is provided.

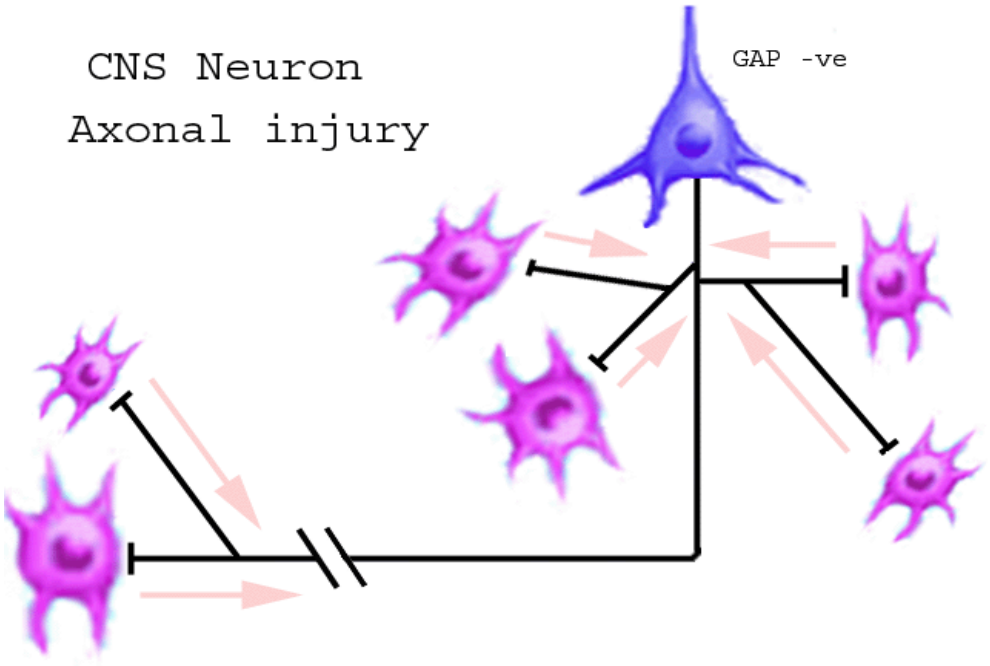

Figure 1.3: The maintenance of retrograde repression in CNS neurons by multiple sustaining collaterals. Distal axotomy does not interrupt retrograde repression from local collaterals.

These observations also suggest a possible contribution of retrograde repression in the observed differences in the regenerative capabilities of CNS and PNS neurons. While most PNS neurons elaborate one primary axon with a terminal arborization at its target site and few collateral branches, many CNS neurons possess highly complex axon trees with collateral branches to both local and distant targets, none of which may be considered a primary branch over any of the others. Thus, CNS neurons may receive retrograde repressive signals from more than one, possibly all, of their many axon target sites. A transection of any one of these branches would not result in any change in their levels of GAP expression because retrograde repression is maintained by the remaining intact collateral branches. This scenario is illustrated in figure 1.3.

In the few CNS neuron populations that have been observed to be capable of up-regulating GAP expression in response to axotomy with limited regeneration into permissive environments, up-regulation of GAP expression and regeneration of axons occurred only when the axotomy was performed close to the neuron cell body. More distal axotomies did not result in any up-regulation of GAP expression or any evidence of regenerative capacity. (Richardson et al., 1984; Doster et al., 1991; Fernandes et al., 1999) This contrasts with PNS neurons, where the distance of the axotomy to the neuron cell body has no effect on the subsequent up-regulation of GAPs. (Liabotis and Schreyer, 1995) These results may be interpreted as supporting the idea that retrograde repression from multiple axon collaterals continues to inhibit GAP expression in CNS neurons after axotomy, with only axotomies proximal to the first collateral axon branch being capable of inducing an up-regulation of GAPs. The ability to up-regulate GAPs after proximal axotomy has been observed in only some CNS neuron populations. Proximal axotomy experiments are not technically feasible for many CNS neurons. Also, sustaining collaterals do not provide only retrograde repressive signals. Neurons also receive trophic factors from their axon collaterals, and many CNS neurons respond to proximal axotomy with atrophy (Dusart et al., 2005) and/or apoptosis.

1.6 HYPOTHESIS PART ONE

In this research project, our aim is to investigate the retrograde repression of GAP expression in the adult CNS. If the idea of sustaining collaterals is correct, and

retrograde repression operates in the CNS along similar lines to what is known for the PNS, except that multiple axon collaterals provide multiple sources of retrograde repressive signal at all times, then it should be theoretically possible to induce the up-regulation of GAPs by completely blocking the retrograde repressive signal from all collateral target sites.

In order to accomplish this experimentally, it would be necessary to identify and experimentally access all the collateral target sites of a given neuron population. Physical axotomy of all axon collaterals of a diverse axon arborization would be problematic in an *in vivo* model, so some biochemical means of blocking the retrograde signal would be preferable. In the most ideal situation, the actual retrograde repressor itself would be known and could be targeting specifically.

1.7 THE TRANSCALLOSAL NEURON AS AN EXPERIMENTAL MODEL

Neurons which project axons across the corpus callosum to the contralateral hemisphere are found throughout the mammalian neocortex. The vast majority of these neurons, known collectively as callosal or transcallosal neurons, (TCN) are prototypical pyramidal projection neurons. Their cell bodies reside primarily in layer III of the neocortex in primates, and layers II/III and V in rodents.(Conti and Manzoni, 1994; Swanson, 2003)

The axons of transcallosal neurons typically form synapses on the dendrite spines of other pyramidal neurons, also primarily within cortical layers II and III.(Conti and Manzoni, 1994) It is possible that transcallosal neurons are among the targets of their own contralateral counterparts. In addition to their contralateral projections across the corpus callosum, transcallosal neurons also possess multiple axon collaterals to ipsilateral targets. Importantly, transcallosal neurons within the parietal cortex project their distal axon collaterals to homologous areas of the contralateral cortex, while their ipsilateral collaterals are largely confined to immediately local and nearby areas of the ipsilateral neocortex.(Cracco et al., 1989; Thomson and Bannister, 1998; Swanson, 2003) For example, transcallosal neurons in the primary somatosensory cortex possess some ipsilateral collaterals projecting into the neighbouring primary motor cortex.(Orihara et al., 1997)

During development, TCNs, like all neocortical pyramidal neurons, are born in the ventricular zone and migrate outwards along radial glia. (Bielas et al., 2004) Their axons first project ventrally under the influence of semaphorins, then turn medially beneath the cingulate cortex. From the cingulate cortex they turn ventrally to reach the corticoseptal boundary, where they make another sharp medial turn to cross the midline. Within the contralateral hemisphere, they pass beneath the contralateral cingulate cortex and then onwards to their final neocortical targets. Collateral branches generally grow out from the main axon at right angles.

A number of midline glial structures appear to provide guidance cues for the midline crossing.(Richards, 2002) Axons from the cingulate cortex may act as pioneering fibers for the majority of the other transcallosal axons. These cingulate neurons project axons both medially across the midline and laterally into the ipsilateral neocortex, and their projections develop earlier than those of other transcallosal neurons, which have been observe to fasciculate with the lateral cingulate fibers to reach the cingulate cortex, and then follow the medial cingulate fibers across the midline to form the corpus callosum.(Richards, 2002)

As previously mentioned, the transcallosal axons are among the telencephalic midline crossing projections that appear to depend on the function of GAP-43. Agenesis of the corpus callosum is a fairly common developmental abnormality, associated with multiple genetic and structural defects. In most cases, transcallosal axons reach the midline but fail to cross, (Richards, 2002; Shen et al., 2002) indicating that failure of midline crossing is the primary deficit underlying these abnormalities.

Several neurosurgical procedures in humans involve the division of portions of the corpus callosum. For example, anterior corpus callosotomy is indicated for seizure control in some circumstances. Animal studies have shown that transcallosal neurons survive for long periods after callosotomy, and display essential no evidence for any morphological or metabolic alterations related to either atrophy, degeneration, or axon regeneration in response to callosotomy.(Orihara et al., 1997) It generally appears that transcallosal neurons continue to function normally for the most part after callosotomy, supporting the idea that they participate in important ipsilateral circuits and possibly receive sustaining trophic factors from their ipsilateral targets.

For the purposes of investigating retrograde repression in central nervous system neurons, transcallosal neurons offer a number of advantages. Within the rodent parietal cortex, the location of both their ipsilateral and contralateral axon target sites are known, being within layers II/III and V of the ipsilateral neocortex in the local area around the neuron cell bodies, and the homologous area contralaterally. These areas are readily accessible near the surface of the brain for the application of biochemical agents through infusion or injection. The corpus callosum provides a convenient location for surgical lesioning of the contralaterally projecting axons, with callosotomy being a well tolerated intervention producing minimal morbidity in experimental animals.

1.8 FGF-2 AS A POTENTIAL RETROGRADE INHIBITOR

Basic fibroblast growth factor, bFGF or FGF-2, is a potent neurotrophic factor first isolated in mature pituitary and the mature brain,(Gospodarowicz, 1974) and subsequently found to be widely distributed in the developing and adult CNS. FGF-2 mRNA expression has been detected throughout the developing CNS, with the highest levels found in the colliculi, cortex , thalamus, and olfactory bulb.(Ernfors et al., 1990) In the mature brain, FGF-2 mRNA has been detected in cortex, hippocampus, hypothalamus, and pons.(Emoto et al., 1989)

FGF-2 is known to have effects on both neuron and glial differentiation. It has been shown to inhibit the differentiation of OP precursor cells into oligodendrocytes, (Zhou and Armstrong, 2007) and regulate neural stems cells during development in a complex manner. Depending on which of a number of known receptors are activated, FGF-2 can promote self-renewal, differentiation, or apoptosis of cortical neural stem cells.(Maric et al., 2007)

FGF-2 is mitogenic for a variety of mesenchyme neuroectoderm derived cell types, including fibroblasts and glial cells,(Gospodarowicz, 1974; Davies et al., 1997a) and has been demonstrated to promote neuron survival.(Morrison et al., 1986; Johnson-Farley et al., 2007) It is also known to stimulate the expression and secretion of nerve growth factor by astrocytes in culture.(Yoshida and Gage, 1991)

Levels of FGF-2 in mature CNS are persistently increased after cortical injury. FGF-2 mRNA expression rises in areas adjacent and ipsilateral to the injury site, and can

be detected in ependymal cells of the lateral ventricle, and selected cells in the cortex and hippocampus. FGF-2 can also be found abundantly in the microglia and macrophages bordering the injury site.(Frautschy et al., 1991)

There is evidence that FGF-2 interacts with GAP-43. Activation of the FGF receptor by FGF-2 results in phosphorylation of GAP-43 in cerebellar granule cells(Meiri et al., 1998) and hippocampal neurons.(Tejero-Diez et al., 2000) The effects of FGF-2 on GAP expression and axon growth vary among neuron populations. There is evidence for both negative, positive, and mixed influences. Unpublished experiments in our laboratory have suggested a possible role for FGF-2 as a retrograde inhibitor in DRG neurons. DRG neurons normally express high levels of GAP-43 when grown in culture, but GAP-43 expression was greatly diminished if the neurons were cultured in the presence of FGF-2.

In contrast to the above, FGF-2 promotes axon regeneration in frog retinal ganglion cells. Application of FGF-2 to transected optic nerves increases GAP-43 expression in the frog retina and affects the distribution of the GAP-43 protein.(Soto et al., 2003) During development, FGF-2 may also be involved in providing pathfinding cues guiding retinal ganglion cell axons into the tectum. (Kalil et al., 2000) In rodent motoneurons, treatment with FGF-2 after sciatic nerve transection resulted in a decrease in the usual up-regulation of another growth associated protein, alpha-CGRP, but had no effect on GAP-43. Cultured embryonic motoneurons responded to FGF-2 by down-regulating alpha-CGRP but up-regulated GAP-43.(Piehl et al., 1998)

FGF-2 has been shown to promote collateral axon branching in cortical pyramidal neurons through a combination of negative and positive effects. Initially, FGF-2 appears to decrease the mobility of the primary growth cone, helping in triggering the pausing and subsequent increase in growth cone size that precedes the development of a collateral branch. Later, FGF-2 promotes the extension of those collateral branches.(Kalil et al., 2000; Szebenyi et al., 2001)

For transcallosal neurons, the available evidence suggests that FGF-2 has an inhibitory effect in this neuron population. Experiments with cultured transcallosal neurons at different stages of development found that when FGF-2 was added to the culture medium, transcallosal neurons in late embryonic and early postnatal stages

displayed a marked decrease in neurite outgrowth, axon length, number of minor processes and dendrites, and complexity of growth cone filopodia. (Catapano et al., 2004)

1.9 HYPOTHESIS PART TWO

Based on the preceding evidence, we formulate for testing the following hypothesis: the expression of the growth associated protein GAP-43 is tonically inhibited by target-derived retrograde signals in the CNS in a mechanism similar to that thought to operate in the PNS. In transcallosal neurons, this retrograde repression is provided by target factors from both its contralateral and ipsilateral axon collaterals. One of the substances involved in providing this retrograde inhibitory signal for transcallosal neurons is FGF-2. Accordingly, it should be possible to produce an up-regulation of GAP-43 expression in this neuron population by simultaneously interrupting FGF-2 signaling from both distal contralateral and local ipsilateral target sites. Alternately, a unilateral interruption of either the distal or the local target sites in isolation should not produce any increase in GAP-43 expression, or at least an increase of lower magnitude.

METHODS

2.0 OVERVIEW OF EXPERIMENTAL PLAN

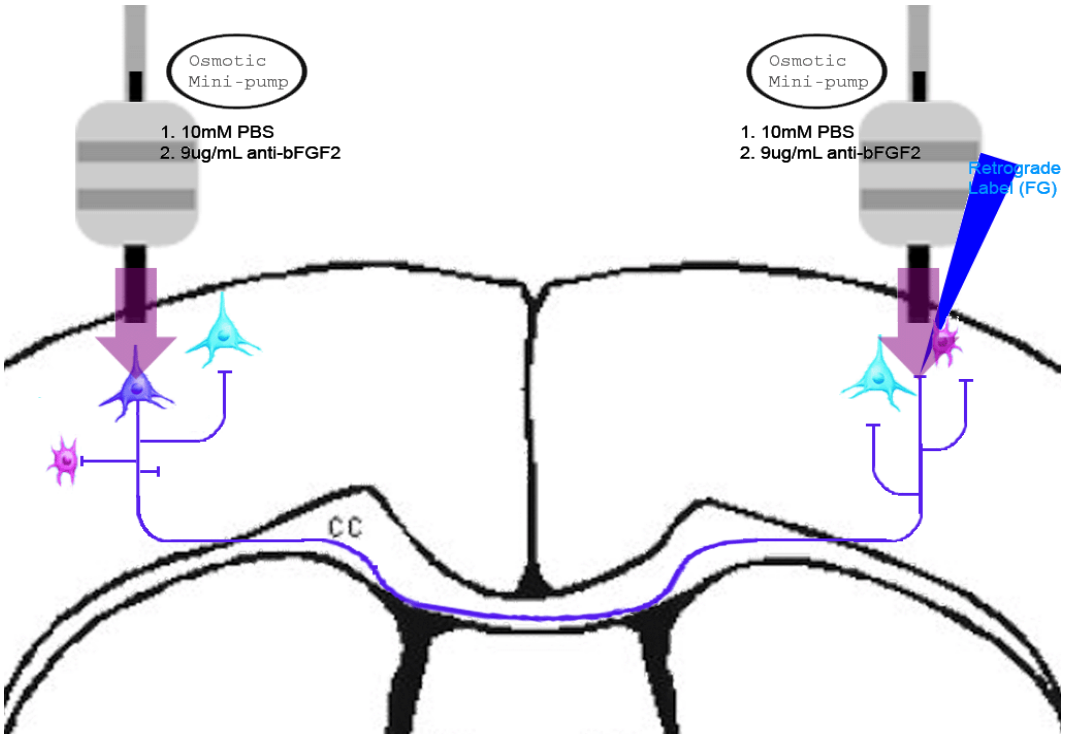

Figure 2.1: Schematic of bilateral infusion experiment. Anti-FGF-2 is infused into the local ipsilateral and homologous distal contralateral axon target sites of fluorescently labeled transcallosal neurons. Results are compared with controls infused with saline buffer alone.

Transcallosal neurons in the rat parietal cortex were identified using the retrograde fluorescent marker Fluorogold™ (Fluorochrome, Denver, CO). In order to determine the role that FGF-2 plays in the retrograde repression of axonal regeneration in transcallosal neurons, adult rats were treated with a bilateral, ipsilateral, and contralateral infusion of a function blocking antibody to FGF-2 (rabbit IgG, Sigma, K50 = 5.0 ug/mL), administered via an implanted osmotic mini-pump, for a period of 7 days. Levels of GAP-43 mRNA in identified TCNs exposed to bilateral, ipsilateral, and contralateral

antibody infusion were compared with control animals receiving infusions of buffered saline vehicle alone (figures 2.1, 2.2, and 2.3).

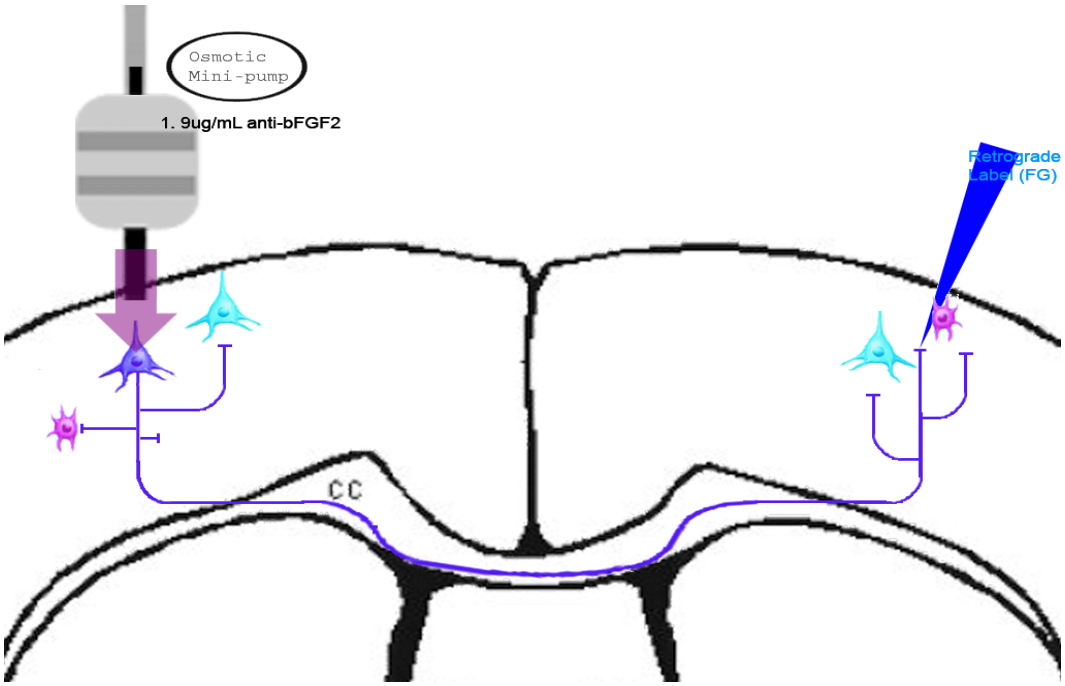

Figure 2.2: Schematic of ipsilateral infusion experiment. Anti-FGF-2 is infused into the local ipsilateral area to block FGF-2 signaling from local axon target sites only.

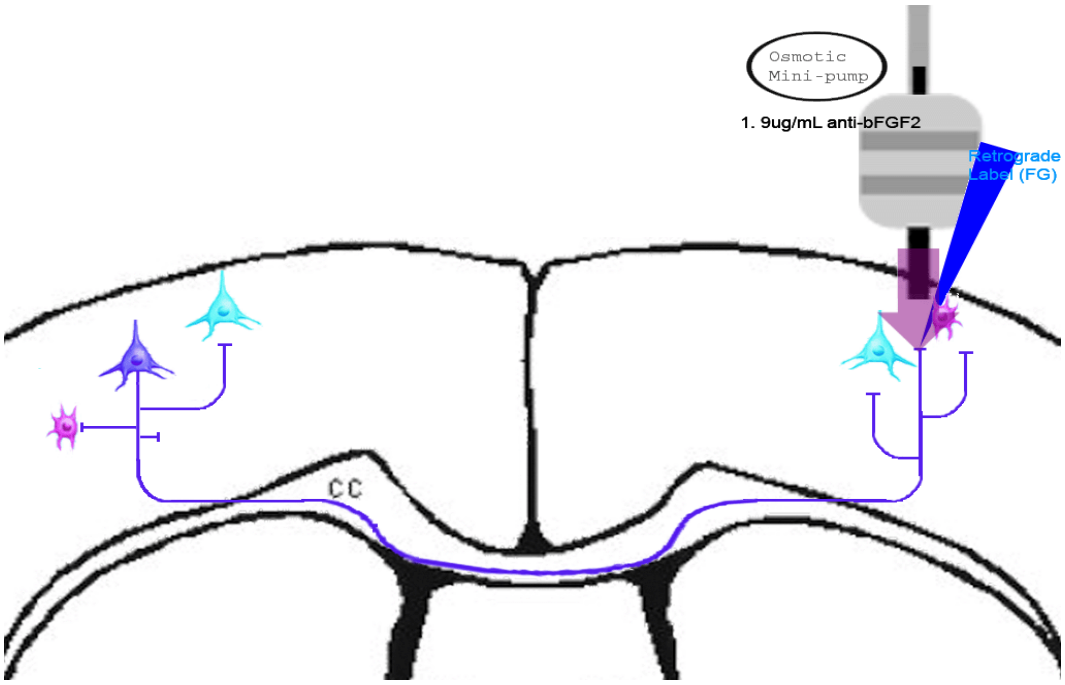

Figure 2.3: Schematic of contralateral infusion experiment. Anti-FGF-2 is infused into the homologous contralateral target site to block FGF-2 signaling from distal axon target sites only.

To investigate whether or not a blockade of ipsilateral retrograde FGF-2 signaling can promote GAP-43 expression after axon injury in transcallosal neurons, a stereotactic sectioning of the anterior half of the corpus callosum was performed. Callosotomized animals treated with concurrent ipsilateral infusion of the function blocking antibody to FGF-2 were compared to animals subjected to callosotomy alone. Both these groups were also compared with the control, bilateral, ipsilateral and contralateral antibody infusion groups already described (figure 2.4).

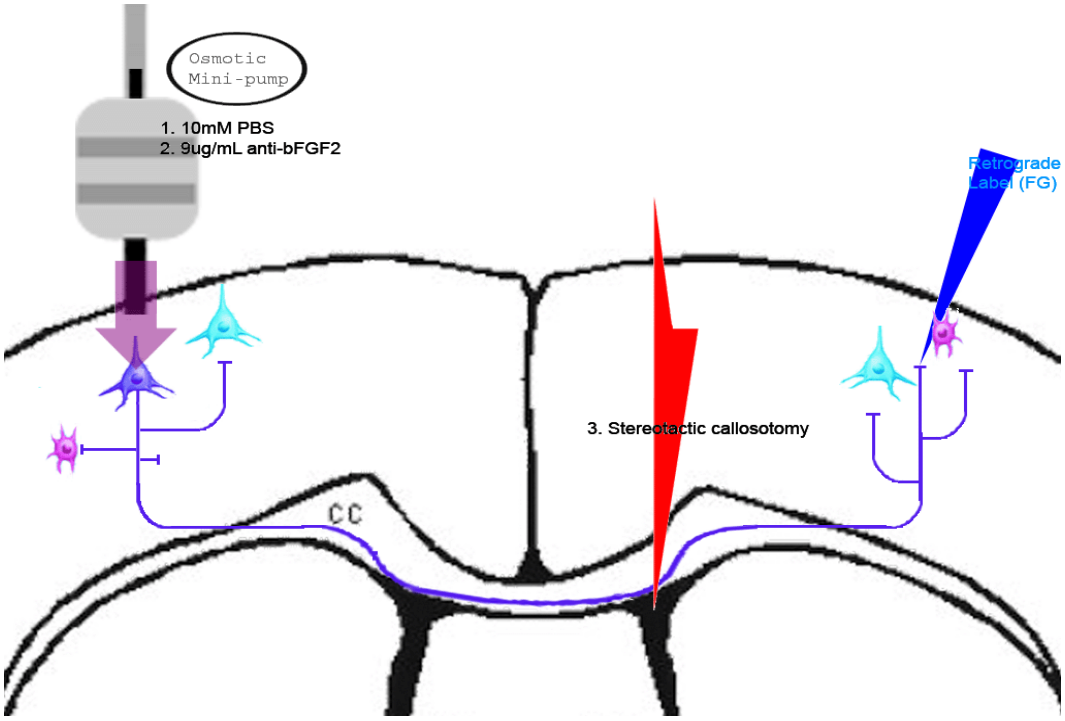

Figure 2.4: Schematic of callosotomy experiment. Stereotactic anterior callosotomy interrupts retrograde signaling from distal contralateral axon targets, while local FGF-2 signaling from ipsilateral axon target sites blocked by an infusion of Anti-FGF-2, or left intact by a sham infusion of saline vehicle.

In each experimental group, GAP-43 expression was quantified through in-situ hybridization of GAP-43 mRNA with a radioactive probe. Preliminary experiments were performed to determine what dose of anti-FGF-2 to use for the main experiments and to refine the technique for the stereotactic callosotomy to provide reliable transection of the corpus callosum with a minimum of bleeding and surgical morbidity.

2.1 CONFIRMATION OF ACTIVITY AND SPECIFICITY OF THE ANTIBODY

2.1.1 Western Blot

The specificity of the anti-FGF-2 for FGF-2 in rat cortex was confirmed using a Western Blot. Rat cortex was harvested from a naïve adult rat and homogenized in Lysis Buffer (20 mM HEPES pH 7.5, 50 mM KCl, 10% glycerol, 0.5 mM RNase-free EDTA, 0.5 mM RNase-free EGTA, 1 mM DTT, 1X Sigma anti-protease cocktail). The suspension was spun down at 4000 rpm for 15 minutes, the supernatant collected, and spun again at 14 000 rpm for 20 minutes. A protein bioassay was used to determine the total protein concentration (3.9 mg/mL).

50 ug of total rat cortex extract protein was loaded onto a 12% Polyacrylamide gel, along with 75 and 100 ng of human recombinant FGF-2 in separate lanes and run at 120V. The proteins were then transferred from the gel to nitrocellulose membrane at 120V for 1h. The nitrocellulose membrane was incubated in blocking solution (3% Horse Serum in PBST with 0.5% BSA and 3% milk) for 1 h. A 1:5000 dilution of the antibody was then added to the blocking solution, and incubated overnight at 4°C.

The nitrocellulose membrane was washed in PBS for 10 minutes x3, then incubated with secondary antibody (IRDye 800CW Donkey anti-Rabbit, LI-COR, 1:10 000 dilution in blocking solution) for 1 h. The membrane was washed with PBST for 10 minutes x2, then PBS for 10 minutes x1. The membrane was then scanned with a LI-COR scanner.

2.1.2 Cell Proliferation Assay

The function blocking activity of the anti-FGF-2 used in our experiments was confirmed using a cell proliferation assay. 3T3 fibroblasts were grown in a 75 mL tissue culture flask in DMEM medium containing 5% Horse Serum (5% HS) at 37°C and 0.5% CO_2 for 24 to 48 h to confluence. Cells were washed with PBS, and detached by incubating at 37°C in 5 mL Versine containing 0.5 mL 1% trypsin for 2 to 3 min. The cell suspension was transferred to a 15 mL eppendorf tube. The reaction was stopped with 1 mL of 5% HS, and medium was added to a total volume of 10 mL. The cells were

spun into a pellet at 800 rpm for 5 minutes, and resuspended in culture medium and counted using a hemocytometer.

The growth proliferation assay was modified from previously published techniques.(Gospodarowicz, 1974; Davies et al., 1997a) Low densities of cells, 2 x 10^3 to 1 x 10^4 cells/well, were plated onto a 96-well microplate and incubated in DMEM medium with 100 uL 0.5% Horse Serum for 48h, at which point the cells had reached 50% to 70% confluence. Varying concentrations of human recombinant FGF-2 (Sigma) were incubated for 2 h with varying concentrations anti-FGF-2, both diluted in DMEM with 0.5% bovine serum albumin to act as a blocking agent, and then 20 uL of the combined solution was added to the 96-well microplate. The cells were then incubated with the FGF-2 and antibody for a total of 24 h further. The cells were pulse labeled with BrdU for final 12 h of the incubation.

The culture medium was then removed by gently inverting the microplate, and the cells were fixed in 100% methanol at -20°C for 1 h. The wells were washed with PBS x1 then incubated in 2N HCl with 0.5% TritonX at room temperature for 1 hour to open the nuclear membrane. Afterwards the cells were washed with PBS, pH 8.4 for 5 minutes x1, then PBS, pH 7.4 for 10 minutes x 2. The cells were incubated in blocking solution (3% Horse Serum in PBST with 0.5% bovine serum albumin (BSA)) at room temperature for 1 h.

The cells were incubated for 1 h with primary antibody (mouse anti-BrdU, Sigma, 1:100 dilution in blocking solution), and then washed with PBS for 5 minutes x3. The cells were then incubated with secondary antibody (IRDye 800CW Goat anti-mouse, LI-COR, 1:500 dilution in blocking solution) for 1 h, then washed with PBST for 5 minutes x2, then PBS for 5 minutes x1. The plate was read using a LI-COR fluorescent scanner and quantified using Odyssey software.

2.2 SURGICAL PROCEDURES

2.2.1 Animal Care and Anaesthesia

All procedures using animals were approved beforehand by a local animal care committee. Male Sprague-Daley rats weighing between 250 and 300 g were obtained

from Charles River and housed in our laboratories in accordance with the standards of the Canadian Council on Animal Care.

Anaethesia was induced with halothane inhalation (2% in 100% oxygen) administered in an induction chamber and maintained via a respiratory mask fitted over the animal's snout. Animals were monitored throughout all procedures by observing the rate and depth of respiration, the colour of mucous membranes and paw pad skin, and pinching the hind paw with forceps. Halothane levels were adjusted as necessary during procedures, with maximum levels never exceeding 2.5%. Post-operative analgesia was provided by subcutaneous injections of 0.5% buprenorphine q8h as needed on post-operative day 1.

Surgical instruments were heat sterilized prior to and after each procedure. Aseptic technique was used in all procedures.

2.2.2 Labeling of Transcallosal Neurons with the Retrograde Fluorescent marker Fluorogold™

After the administration of halothane anaesthesia, the animal was placed on an electric heating pad to prevent hypothermia. After an adequate depth of anaesthesia was confirmed by pinching the hind paw, the head of the animal was immobilized in a stereotactic frame (David Kopf Instruments). The hair over the head was shaved and the skin was sterilized with isopropyl alcohol. Sterile paper towels were laid over the animal's body and the working area to establish a sterile field.

A 15 to 20 mm incision was then made with a scalpel in the skin at the midline on the top of the head, starting just behind the level of the eyes and extending back beyond the level of the external auditory meatus. The periosteal tissue was dissected free to expose the cranium underneath. A small self-retaining retractor was used to hold back the skin edges. Using an operating microscope, the coronal and sagittal sutures, and the Bregma point were identified, and freed of any remaining attached periosteal tissue. If any bleeding was encountered it was soaked up with strips of sterile filter paper or a small amount of sterile cotton and pressure applied until hemostasis was restored.

Using the stereotactic frame, a point -0.8mm (caudal in the rostro-caudal plane) to Bregma and 4.0mm to the right of midline (as marked by the sagittal suture) in the

medial-lateral plane was identified, where a burrhole roughly 2 to 3 mm in diameter was drilled through the skull until the underlying dura was exposed, as described in figure 2.5 The dura was then punctured with the tip of a 27 gauge syringe to expose the surface of the brain over the parietal cortex.

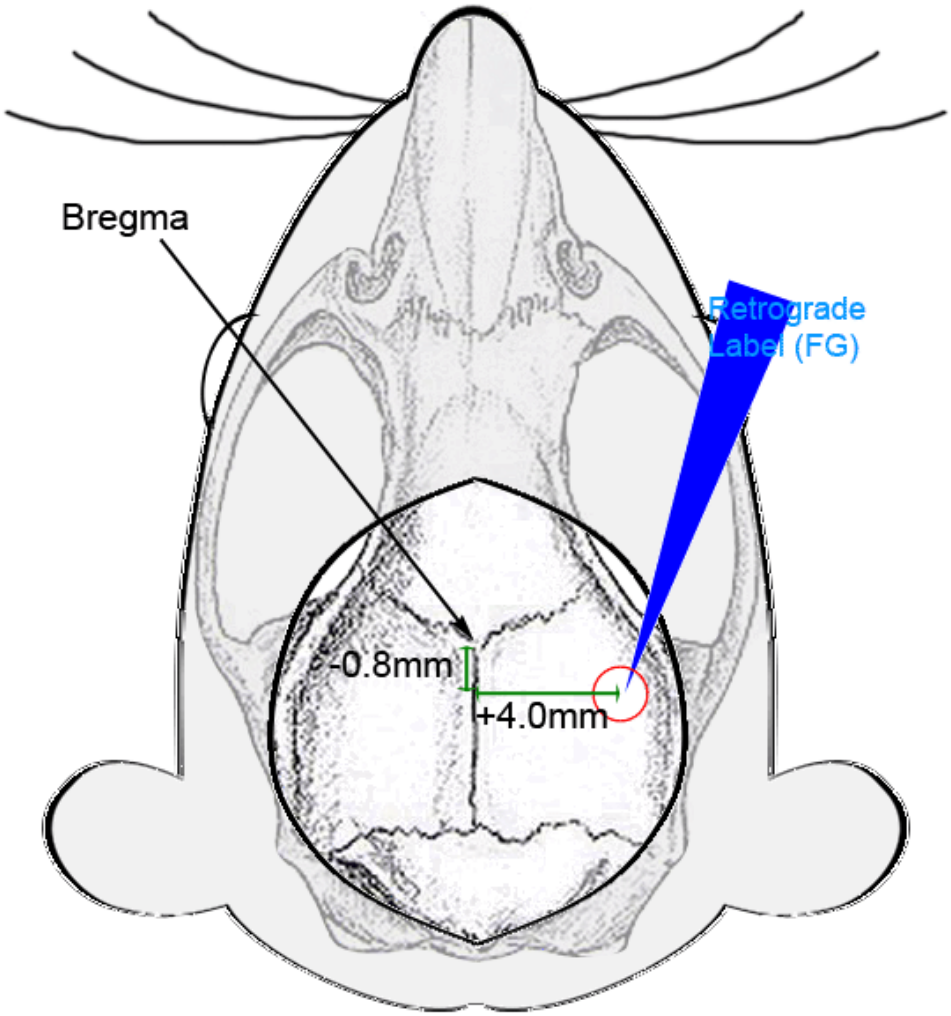

Figure 2.5: Retrograde labeling of left parietal transcallosal neurons. A burrhole was made in the skull overlying the right parietal cortex at the specified coordinates, and the fluorescent retrograde dye was injected into the contralateral axon target site of the target neuronal population.

0.4 uL of a 4% solution of Fluorogold™ (FG), stored in a lightproof container, was drawn up using a glass micropipette, which was attached to the arm of the stereotactic frame. The retrograde fluorescent dye was then delivered using the micropipette at the previously identified point, -0.8 mm caudal and 4.0 mm to the right relative to Bregma, into the neocortex at a depth of 2.5 mm below the pial surface. This

depth was calculated to deliver the dye near the junction between cortical layers IV and V. The dye was injected slowly over about 2 minutes at an average rate of about 0.005 uL per second.

After the dye injection was complete, any obvious bleeding was stopped by the application of gentle pressure. 4.0 Silk suture was used to close the skin. The surgical site was cleaned with sterile water, and inhalation anaesthesia was discontinued. The animal was placed on a paper towel in a fresh cage and observed until it was awake and active. We waited seven days prior to any additional surgical procedures to allow for sufficient labeling of the contralateral transcallosal neurons through retrograde transport of the FG.

2.2.3 Preparation of the Osmotic Mini-pumps

We used Alzet Osmotic Mini-pumps with brain infusion kits (Model 2001, Durect, Cupertino, CA) for our experiments. Solutions were prepared 24h prior to implantation. All preparations were done under sterile conditions. The attachment knob on each brain infusion cannula tip was glued to a cut piece from the base of a sterile standard medium sized pipette tip. The length of the cannula tip was set to 3.0 mm, the shortest available length. The cannula tips were then attached to the provided tubing, which were cut to 7.5 cm in length, and attached at the cut end to the mini-pump reservoir cap. The cannula tip, tubing and reservoir cap, along with the reservoir, were weighed while empty. The mini-pump reservoir and cannula tubing were then filled completely using the provided 1 mL syringe and blunt syringe tip and attached together, taking care to avoid bubble formation in the cannula tubing. Mini-pumps were filled with test solution (9 ug/mL solution of anti-FGF-2 in PBS, 9 ug/mL rabbit serum IgG in PBS, or with the vehicle alone).

After filling, the mini-pump reservoir and attached brain infusion cannula were re-weighed once filled to ensure that the filled weight was at least 250 mg more than the empty weight. The mini-pump reservoir and attached cannula were then placed in a sterile beaker and submersed in sterile phosphate buffered saline, and incubated at 37.0°C for 24 h.

2.2.4 Implantation of the Osmotic Mini-pumps

Experimental animals which had their left parietal transcallosal neurons labeled 7 days prior were once again anaesthesized with halothane and placed in the stereotactic frame. The skin over the top of the skull was shaved again, and the shave extended over the shoulders and back. The whole area was prepped as before with isopropyl alcohol and the sterile field was set up as before.

The silk stitches closing the prior incision were cut with a pair of fine scissors and the previous incision in the midline of the scalp was reopened. The incision was extended caudally 2.0 to 3.0 cm with a scalpel through the skin, and a subcutaneous pocket was created by blunt dissection using a pair of scissors. The mini-pump reservoir was then placed inside the subcutaneous pocket.

The coronal and sagittal sutures and the Bregma point were re-exposed, with any scar tissue or blood clot from the prior surgery removed with blunt dissection. Scar tissue and other debris was removed from the right sided burrhole where the FG had been imjected until the pial surface was once again visible. Prior to placement of the mini-pump brain cannula, the skull was thoroughly dried with a cotton swab.

A 3.0 mL syringe was attached to the stereotactic frame arm, and the cut piece of pipette tip base that had been glued to the mini-pump attachment knob was screwed firmly into Luer hub at syringe end. The implantation of the mini-pump brain cannulae, described in figure 2.6, was performed with the aid of the operating microscope. For contralateral mini-pump implantations, the mini-pump brain cannula was placed into the brain at the coordinates -0.8 mm to Bregma in the rostral-caudal plane and 4.0mm in the medial-lateral plane on the right side, through the same burrhole and in the same cortical location as the FG had been injected previously.

For ipsilateral minipump implantations, a second burrhole was made on the left side of the skull, at the coordinates -0.8 mm to Bregma in the rostral-caudal plane and 4.0mm in the medial-lateral plane to the left side of midline. The dura once exposed was opened once again with the tip of a 27 guage syringe needle and the underlying pia of the left parietal cortex was exposed. The mini-pump brain cannula was then implanted at those same coordinates on the left side.

After the brain cannulae were implanted into the brain, an acrylic glue was squirted liberally over the plastic cannula knob until its entire height was covered in glue. The glue was allowed to dry for 3 to 5 minutes until it was noticeably hardened. After this, the brain cannula was detached from the stereotactic frame arm by cutting off the portion of the knob attached to the pipette tip end screwed into the syringe with a pair of fine scissors.

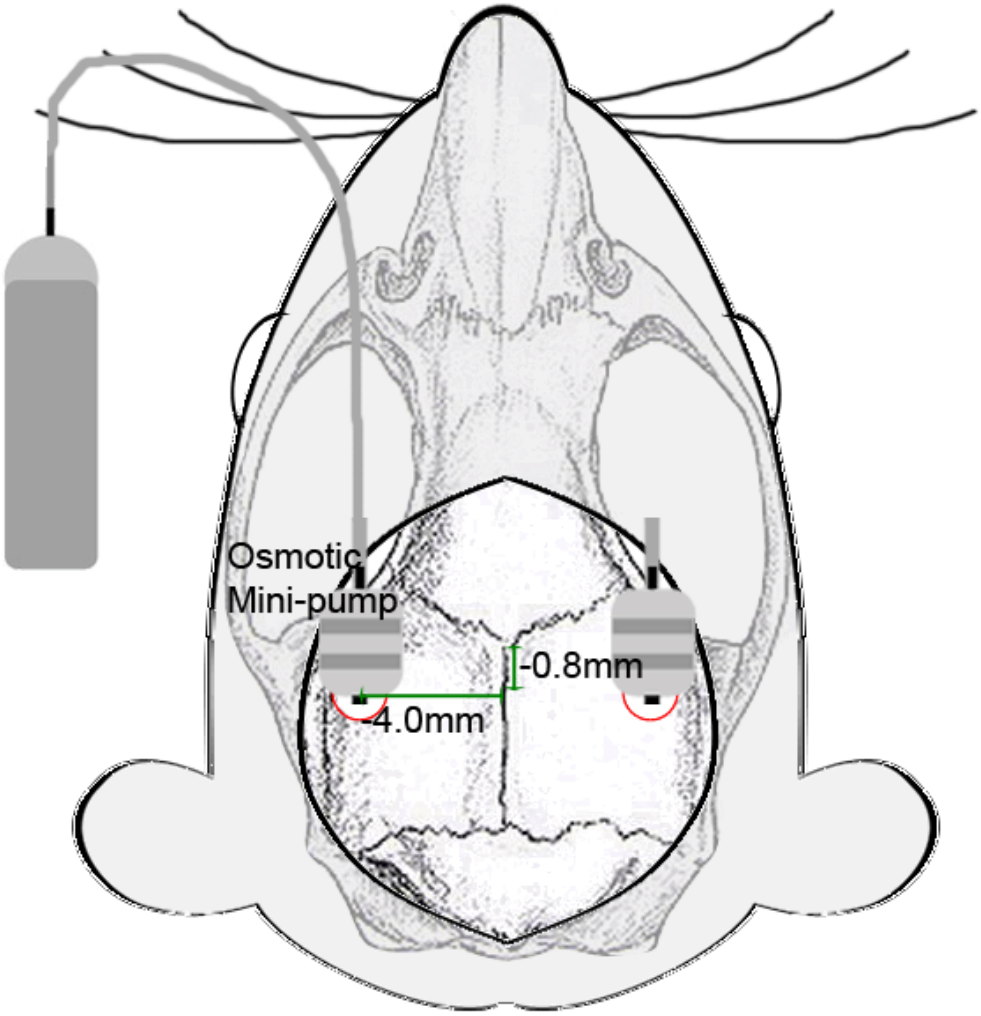

Figure 2.6: Implantation of osmotic minipumps at ipsilateral (left) and contralateral (right) sites. The same burrhole made for retrograde dye injection was re-used for contralateral mini-pump implantation.

For bilateral mini-pump implantations, both mini-pump reservoirs were first placed side by side in the subcutaneous pocket in the animal's back. The ipsilateral mini-pump cannula was implanted in the brain and glued into place first. After detachment of

the ipsilateral brain cannula, the contralateral brain cannula was attached to the stereotactic frame arm via the 3.0 mL syringe and implanted as described above into the contralateral burrhole.

After the acrylic glue was dry and the brain cannulae were securely fixed in place, the scalp was closed over the midline and sutured with 4.0 Silk suture. The animals were recovered from anaesthesia in the same manner as previously described for the injection of retrograde tracer dye.

2.2.5 Stereotactic Callosotomy

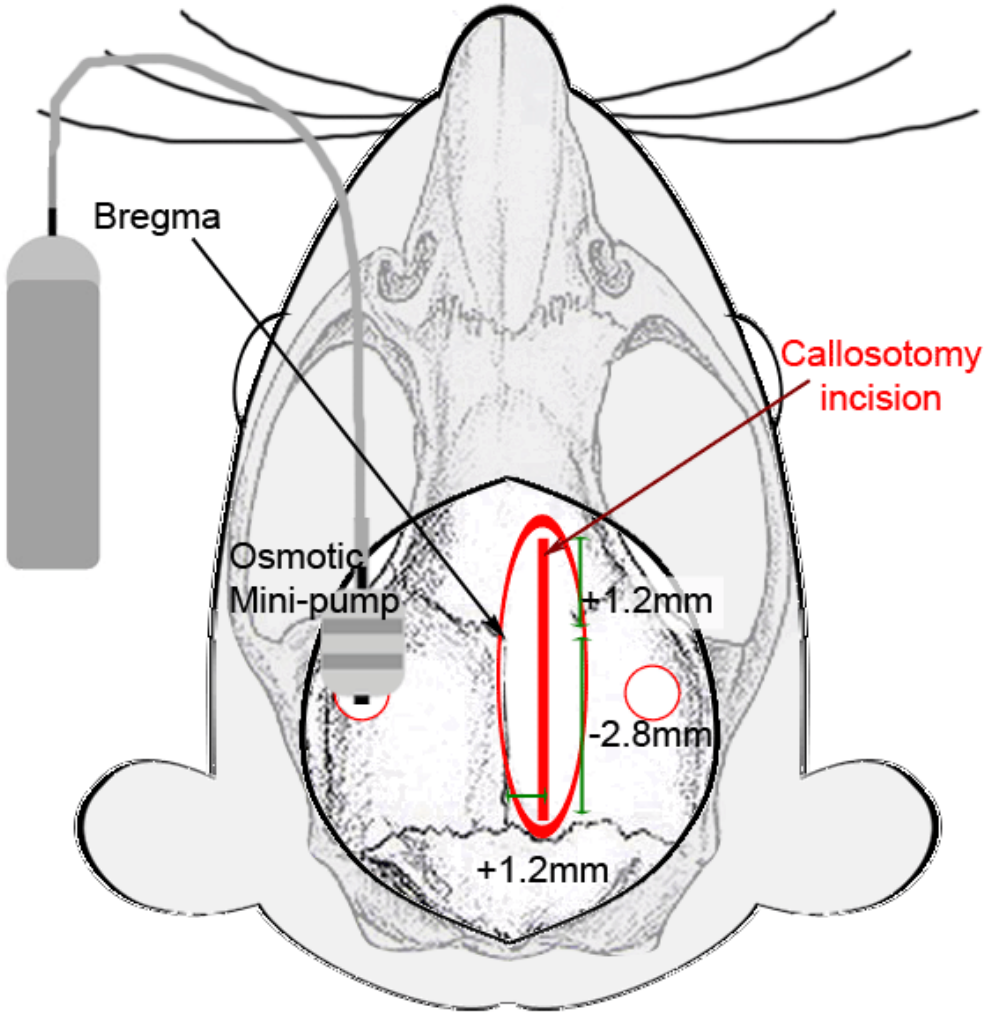

Figure 2.7: Bony removal and cortical incision for stereotactic callosotomy. A slit was drilled through the skull bone 1.2 mm lateral to the midline to accommodate a 4.0 mm cortical incision.

The animals previously injected with FG were anaesthetized and positioned, and the surgical field prepared, as previously described. The midline scalp incision was re-opened after cutting the silk suture. The top of the skull was exposed such that the coronal and sagittal sutures were visible and the Bregma point easily identified. With the aid of the operative microscope, a slit was drilled through the skull 1.2 mm lateral to the midline defined by the sagittal suture on the right side spanning a length of 5 to 6 mm with the approximate center at -0.8 mm to Bregma in the rostral-caudal plane, as illustrated in figure 2.7. The width of the slit was between 2.0 and 3.0 mm. Care was taken not to injure the sagittal sinus, which was visible at the medial margin of the bony exposure.

The technique for the stereotactic callostomy is summarized in figure 2.8. The callosotomy knife was prepared prior to the procedure. It was made by bending 1.5 to 2.0 mm of the tip of a 25 gauge syringe needle at an angle of 135° such that the cutting edges of the needle tip were oriented up and down when the needle tip was pointed forward. The callosotomy knife was screwed onto a 3.0 mL syringe that was attached to the stereotactic frame arm.

After drilling the site in the skull bone, excess bone dust was removed with a cotton swab to expose the dura. The stereotactic coordinates were set to -2.8 mm to Bregma in the rostral-caudal plane and 1.2 mm to Bregma in the medial-lateral plane to the right. The callosotomy knife was lowered until the needle tip just touched the dura. The dura was then opened by carefully moving the callosotomy knife slightly up and down until the needle tip punctured the dura. The needle tip was then advanced slowly by moving the stereotactic frame arm, and the dura split by moving the callosotomy knife up slightly. This was repeated, gradually advancing the callosotomy knife forward until the dura was opened along the entire length of the bony exposure.

After the dura was opened, the needle tip callosotomy knife was repositioned in the rostral-caudal plane to -0.8 mm to Bregma. The callosotomy knife was then lowered slowly to a depth of 4.0 mm, until it cut through the anterior portion of the body of corpus callosum and entered the underlying lateral ventricle. A small gush was CSF was seen coming up out of the cortical incision at this point. The callosotomy knife was then advanced rostrally in the rostral-caudal plane 2.0 mm, to +1.2 mm relative to Bregma,

and lowered to 5.0 mm of depth, cutting through the genu and rostrum of the corpus callosum. The knife was then raised back to a depth of 4.0 mm and moved caudally 4.0 mm, to -2.8 mm to Bregma, and slowly withdrawn to sever the fibres of the middle portion of the body of the corpus callosum. Overall, the anterior third to one half of the corpus callosum was divided in this manner, sparing the posterior third overlying the hypothalamus and avoiding hypothalamic injury.

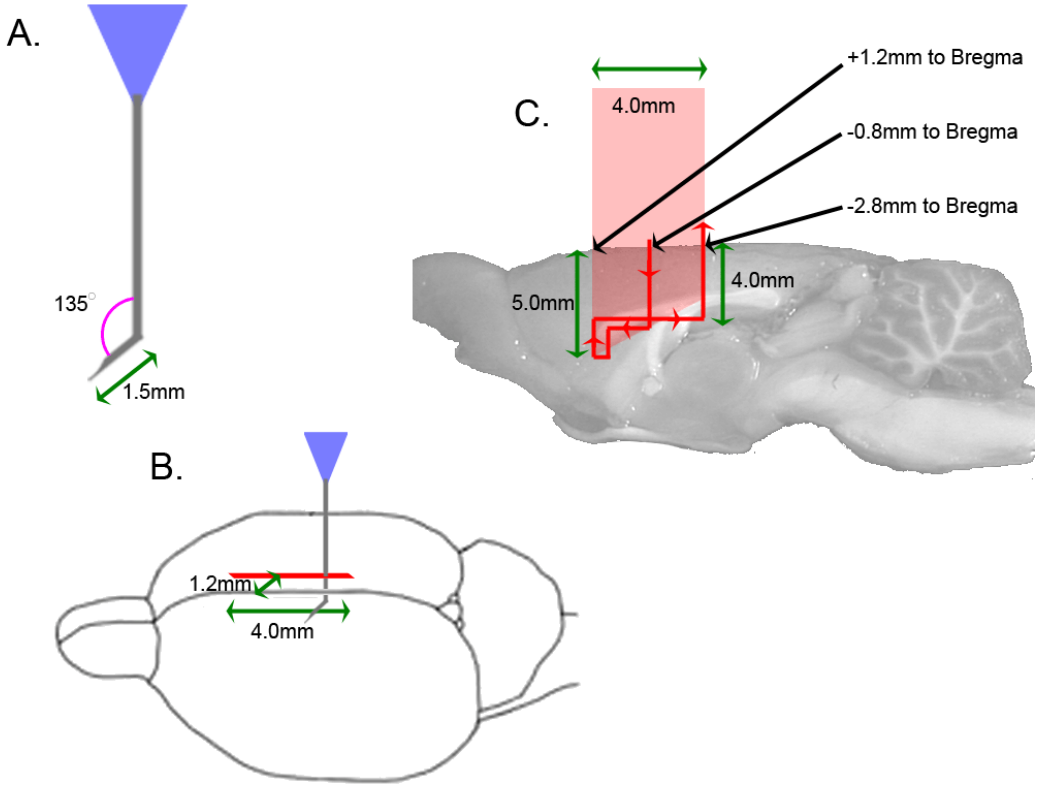

Figure 2.8: Stereotactic callosotomy technique. A) The callosotomy knife was made by bending the tip of a 25 guage syringe needle with the cutting edges facing up and down. B) The cortical incision was made 1.2 mm lateral to midline on the right side and 4.0 mm in length. C) The callosotomy knife was inserted at -0.8 mm to Bregma to a depth of 4.0 mm, advanced 2.0 mm, then lowered 1.0 mm more to cut the genu and rostrum, raised back to 4.0 mm, moved back 4.0 mm to cut the middle portion of the body of the corpus callosum, then withdrawn.

Bleeding from the cortical incision invariably occurred during insertion and after withdrawal of the callosotomy knife. Excess blood was soaked up with strips of filter paper. Gentle pressure was applied with bits of cotton or cotton swabs until the bleeding stopped.

For experiments that combined callosotomy with mini-pump implantation, the burrhole for the ipsilateral mini-pump cannula was drilled on the left side of the skull

prior to performing the callosotomy because drilling the callosotomy slit frequently obliterated or distorted the Bregma point used as a landmark for localizing the burrhole. The actual mini-pump implantation was performed after completion of the callosotomy.

Closure of the operative incision was performed in the same manner as previously described.

2.2.6 Perfusion of Animals and Harvesting of Brain Tissue

Experimental animals were sacrificed 7 days after mini-pump implantation and/or callosotomy. Animals were deeply anaesthetized with intraperitoneal injections of 0.3 mL of Somnotol, and fixed in a supine position using tape. A ventral midline incision was made from the throat to the abdomen. The skin was retracted and a transverse incision was cut through the abdominal wall with a pair of fine scissors, opening the peritoneal cavity. The diaphragm was cut and heavy scissors were used to cut the rib cage on both sides. The base of the sternum was firmly grasped with a hemostat clamp and reflected upwards to expose the heart and lungs.

The heart was grasped with atraumatic forces, and a small incision was made with fine scissors in the inferior pole of the left ventricle. A blunt cannula was inserted into the opened left ventricle until the tip was just within the ascending aorta, and clamped in place with a hemostat. The animal was pre-flushed with 50 mL of cold 100 mM PBS administered through an automatic pump at a rate of 18 to 25 mL / min. A small hole was cut in the right atrium as the flush began to relieve flow. Then the animal was transcardially perfused with cold 4% paraformaldehyde (PFA) in 100 mM PBS until the liver paled and the carcass noticeably stiffened. The volume of PFA used averaged between 200 to 250 mL per animal.

The surgical incision on the back of the skull was reopened and extended. The temporalis muscle was cut free from its attachment on the skull on both sides. A small rongeur was used to removed the C1 posterior arch and skull bone to expose the brain from cerebellum to the cerebral hemispheres up to the beginning of the olfactory bulbs. The spinal cord was cut at the C1 level. A flat spatula was slipped underneath the brain to dissect free the cranial nerves, allowing the entire brain to be lifted from the skull base. The brain was then immersed in PFA and post-fixed overnight at 4°C.

2.3 HISTOLOGIC IDENTIFICATION OF TRANSCALLOSAL NEURONS

Coronal tissue blocks 3 to 5 mm in width spanning the infusion sites were sectioned from the post-fixed brains, cryoprotected by sinking in 10% sucrose for 4 to 8 h, then 20% sucrose overnight on a rotator in a cold room (4°C), and embedded in OCT compound and frozen with dry ice. Coronal sections were cut in a cryostat at 6 um through the injection sites and thawed onto ProbeOn Plus (FisherBiotech, Edmonton, Alberta, Canada) slides.

Each slide was mapped with fluorescent microscopy prior to further processing. Composite maps were assembled from individual low power micrographs of these areas using Adobe Photoshop™ image processing software.

2.4 IN SITU HYBRIDIZATION

In-situ hybridization was validated using control hybridization solutions that included no labeled probe, or labeled GAP-43 probe and 1000X excess unlabeled GAP-43 probe. Both procedures resulted in a hybridization signal no higher than background. When hybridization was performed with labeled GAP-43 probe combined with 1000X excess unlabeled oligonucleotide complimentary for Tα1-tubulin or c-Jun mRNA, the GAP-43 mRNA hybridization signal was not diminished.

The oligonucleotide probe for GAP-43 mRNA and hybridization cocktail solution we used were available and previously used in our laboratory.(Karimi-Abdolrezaee and Schreyer, 2002) The probe was labeled with ^{35}S at the 3' end one day prior to use. The following reagents were mixed in a sterile 1.5 mL Eppendorf tube:

2.5 μL 5X reaction buffer (Amersham, provided with terminal transferase)

8.0 μL autoclaved triple-distilled water

2.0 μL oligonucleotide probe (20 ng/mL)

10.0 μL of [^{35}S]dATP (Dupont NEN, #NEG-034H)

3.0 μL terminal transferase (Amersham #T2230Z)

Gentle pipette mixing was performed after adding each ingredient except for the enzyme. Because the enzyme tended to be very sensitive, it was kept in a freezer container until use, and added quickly without mixing. The mixture was incubated in a

water bath at 37°C for 2.0 h. In order to increase the efficiency of labeling, the first 2.0 μL of terminal transferase was added initially, and the remaining 1.0 μL added after 1.5 h of incubation.

The reaction was stopped by adding 20 μL of tris-HCl and purified using a Chroma Spin-10 column (Clontech). The Chroma Spin columns were prepared as per manufacturer instructions. They were first inverted to suspend the gel matrix, opened, and placed in 2 mL microcentrifuge collection tubes, and centrifuged at 1800 rpm (700G) for 5 minutes. The columns were then placed in fresh microcentrifuge tubes, and the labeled probe reaction mixture was added by drop pipetting it into the center of the gel bed. 5 μL of salmon sperm DNA was added as well. The columns were centrifuged again at 1800 rpm for 5 minutes, and the purified labeled probe was collected afterwards from the bottom of the microcentrifuge tube. A 2 μL sample was withdrawn and the amount of radioactivity was measured by liquid scintillation.

Pretreatment of experimental slides were performed one day after labeling of the oligonucleotide probe was complete, using the following procedure.

1. Slides were removed from the freezer and air dried for 15 minutes
2. Slides were fixed in 4% PFA for 15 minutes
3. Slides were washed 3 X 5 minutes in 10 mM PBS.
4. While the slides were soaking in the PBS, a 20 ug/mL solution of proteinase K was prepared.
5. Slides were treated with the proteinase K for 6 minutes.
6. Slides were post-fixed for 5 minutes in 4% PFA.
7. Slides were rinsed 2 X 5 minutes in 10 mM PBS.
8. Slides were rinsed in DEPC-H_2O for 5 minutes.
9. Slides were dehydrated in alcohol:
    - 1 minute in 70% EtOH.
    - 1 minute in 90% EtOH.
    - 30 seconds in 100% EtOH.

After pretreatment was completed, the slides were left to air dry in a dark place for 30 minutes. Previously frozen hybridization cocktail was thawed in a 43°C water

bath. 100 mL of hybridization solution was prepared for each slide to be hybridized was made using the following recipe.

For each 1 mL, vortexing after adding each component:

900 μL hybridization cocktail.

100 μL salmon sperm DNA (10 mg/mL boiled for 5 minutes, then chilled for 5 minutes immediately before use.)

10^7 cpm ^{35}S-labelled probe.

40 μL 5 M DTT

Hybridization boxes were prepared by lining the bottom with filter paper strips and moistening with triple-distilled water. 180 uL of hybridization solution was then applied to the surface of half of the experimental slides. On top of each of these slides a second experimental slide was placed using a hinge technique, taking care to avoid trapping air bubbles. The hybridization box was taped firmly closed and placed in an oven at 43°C for 14 to 18 hours.

While the hybridization reaction is incubating overnight, 2 to 3 L of 1X SSC was warmed in a 55°C water bath. After the hybridization reaction was complete, two rinsing dishes and two 250 mL beakers were filled with warm 1X SSC. A slide rack was placed in one of the rinsing dishes. The hybridization box was removed from the incubating oven and opened. Each pair of slides was transferred to one 250 mL beaker and gently separated. One slide was placed in the second beaker while the other slide was placed in the slide rack. Then the first slide was transferred to the slide rack, taking care to keep the slides immersed in 1X SSC at all times. When the rack was filled, it was transferred to the second rinsing dish and rinsed for one minute. The slides were then rinsed 4 x 15 minutes in 1X SSC at 55°C in the water bath. The last rinsing dish was then removed from the water bath and allowed to cool for 15 to 30 minutes.

The slides were then dipped 1 second each in 2 dishes of distilled water, 6 X 1 second in 60% EtOH, and 12 x 1 second in 95% EtOH, then left to dry in a dust-free, dark environment.

2.5 QUANTIFICATION OF GAP-43 mRNA EXPRESSION

After in-situ hybridization, slides were developed by dipping in NTB2 nuclear track emulsion (Eastman Kodak, Rochester, NY). To prepare the emulsion, 12 to 15 mL of emulsion cream was added to an equal volume of distilled water in a dark room and melted in a warm water bath for 30 minutes. Slides were dipped back to back, with tissue side facing outwards, and dried on a rack for 3 hours. The dipped slides were transferred to slide boxes in the dark, and each box was wrapped in light proof dark plastic bags, which were left to expose at 4°C. Test slides were developed at one week intervals to determine when the experimental slides were ready for development. Total exposure time for experimental slides ranged from 8 to 28 days. Emulsion coated slides were developed in D19 (Eastman Kodak) for 3 min and fixed in undiluted Rapid Fix (Kodak) for 5 min.

A Zeiss Axioskope microscope set with dark field optics was used to assess the pattern of cell labeling with the GAP-43 mRNA probe. Sections were counterstained with cresyl violet for visualization of silver grains with bright field illumination. Low power bright field composite maps were assembled, as shown in figure 2.11, and the transcallosal neurons were identified by overlaying the fluorescent map of the same section over the bright field map using Adobe Photoshop™ image processing software.

High power images over each identified TCN were captured using bright field optics, and a Northern Eclipse™ subroutine was used to quantify silver grain density within the fluorescently labeled cells. Background silver grain density was determined as the average silver grain density of at least five high power images over cell free areas of the molecular zone overlying the area of analysis. GAP-43 mRNA expression of individual TCNs was calculated by dividing the value returned by the Northern Eclipse™ subroutine for each individual neuron by the average background value.

RESULTS AND ANALYSIS

3.0 DETERMINATION OF ANTIBODY ACTIVITY AND SPECIFICITY

3.0.1 Western Blot

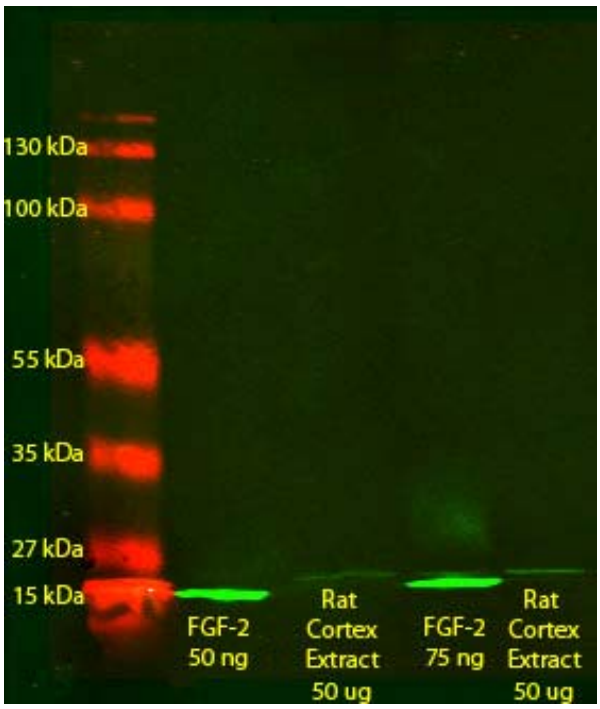

Figure 3.1: Western blot of purified recombinant FGF-2 and rat cortex extract demonstrated specificity of test antibody to FGF-2, and the presence of FGF-2 in adult rat cortex.

To document the specificity of the antibody used to block FGF-2 in the cerebral cortex we determined that the anti-FGF-2 was capable of specifically detecting both purified recombinant human FGF-2, and FGF-2 in tissue extracts of rat cortex. A single band of 17 kDa size was observed in western blots of rat cortex extract, while blots of purified human recombinant FGF-2 detected a primary band of approximately 15 kDa in size, and a faint secondary band of 17 kDa in size. The observed difference may be

attributed to different degrees of heparin complex formation between the two test samples, as well as possible differences in the properties of human and rat FGF-2. The results of the western blot are shown in figure 3.1

3.0.2 Cell Proliferation Assay

We verified that the anti-FGF-2 antibody purchased from Sigma™ possessed function blocking activity as claimed by the manufacturer. Our method was to determine if the antibody would block the ability of exogenous FGF-2 to stimulate the proliferation of cultured 3T3 fibroblasts.

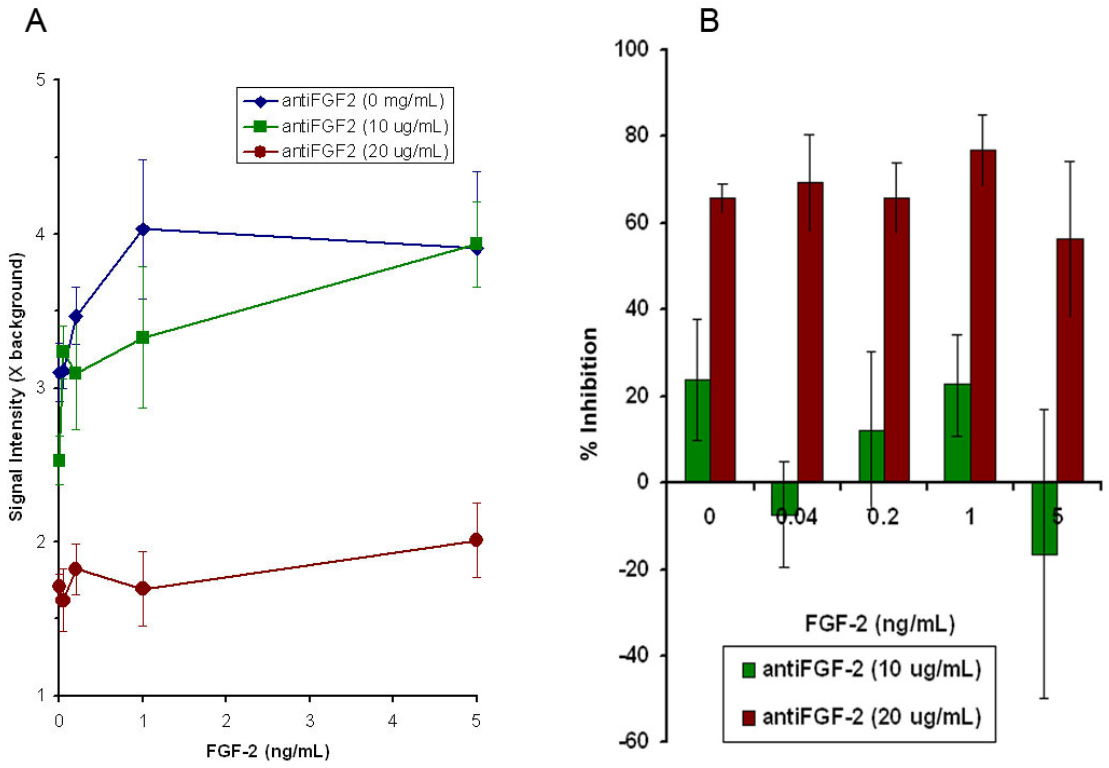

Figure 3.2: Results of a cell proliferation assay of 3T3 fibrobasts grown in the presence of varying concentrations of FGF-2 and anti-FGF-2. A) Increasing concentrations of FGF-2 result in logarithmically higher rates of cell proliferation. B) The presence of 20 ug/mL of anti-FGF-2 substantially inhibits the activity of FGF-2.

The results of the cell proliferation assay are shown in figure 3.1. The rate of cell proliferation of 3T3 fibroblasts grown in DMEM culture medium with 0.5% horse serum initially increased with increasing concentrations of FGF-2, then reached a plateau and

began to fall as concentrations of FGF-2 continued to increase. This finding was consistent with previously reported observations.(Davies et al., 1997a)

The addition of 20 ug/mL of anti-FGF-2 to the culture medium substantially inhibited the proliferation of 3T3 fibroblasts grown in medium containing 0 to 5 ng/mL of FGF-2. Percent inhibition was calculated using the formula % inhibition = (Signal $\text{Intensity}_{\text{experimental antiFGF-2 concentration}}$ − Signal $\text{Intensity}_{\text{no antiFGF-2}}$) / (Signal $\text{Intensity}_{\text{no antiFGF-2}}$ – 1). 20 ug/mL of anti-FGF-2 inhibited 69.4 +/- 11.3 % of the activity of 0.04 ng/mL of FGF-2, 65.6 +/- 8.1% of the activity of 0.2 ng/mL FGF-2, 76.7 +/- 8.2% of the activity of 1.0 ng/mL FGF-2, and 56.3 +/- 18.1% of the activity of the activity of 5.0 ng/mL FGF-2. A percent inhibition of 65.7 +/- 3.6% was also observed even when no FGF-2 was added to the culture medium, and is most likely attributed to the inhibition of the activity of low levels of endogenous FGF-2 present in horse serum, or produced by cells in culture.

3.1 QUALITATIVE OBSERVATIONS

Observations of brain sections under fluorescent microscopy prior to in situ hybridization revealed variable intensity of FG labeling of TCNs between individual animals. Some animals exhibited extremely strong fluorescent labeling where a large number of pyramidal neurons in the parietal cortex took up the fluorescent dye after a contralateral injection of FG, while other animals only had a few fluorescently labeled neurons. In all experimental animals, the homologous nature of the transcallosal projection was confirmed by the observation of abundant fluorescently labeled pyramidal neurons in ipsilateral parietal cortical areas homologous to the contralateral FG injection site. Other areas of fluorescent labeling were also observed in some sections, including far lateral cortical areas, and medial cingulate cortex. These distant areas were not analyzed in our experiments.

An example of a composite fluorescent map of the area of analysis around an ipsilateral mini-pump infusion site is shown in figure 3.3. In all experimental animals, the laminar distribution of labeled neurons in layers II/III and V was readily apparent on fluorescent microscopy. The injection site scar was easily identifiable on most sections.

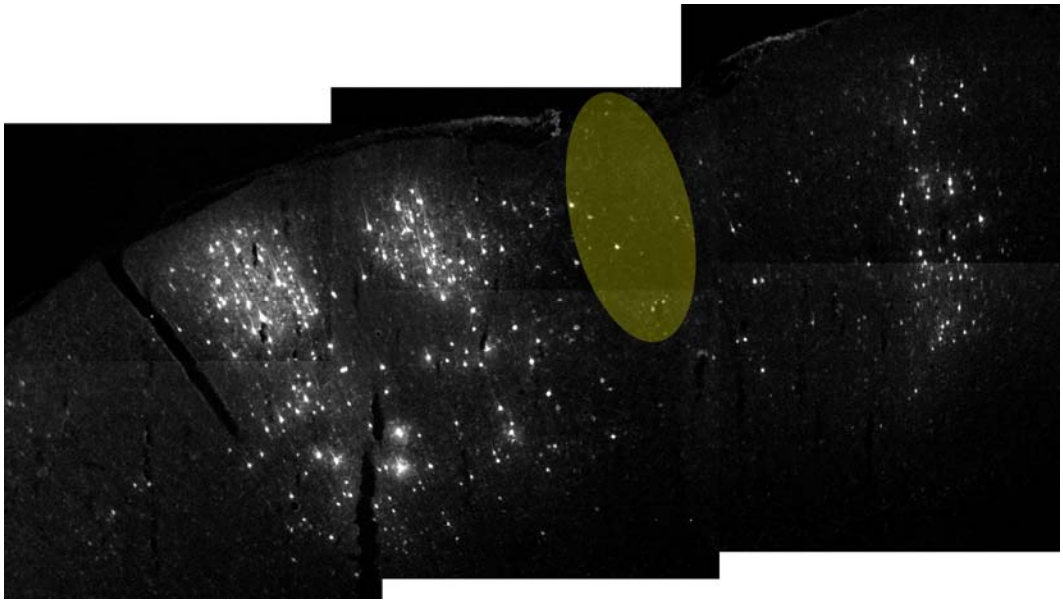

Figure 3.3: Composite fluorescent map of left parietal cortex area homologous to the contralateral FG injection site. Labeled TCNs are found predominantly in layers II/III and V. The yellow oval marks the area of scar around the ipsilateral infusion site.

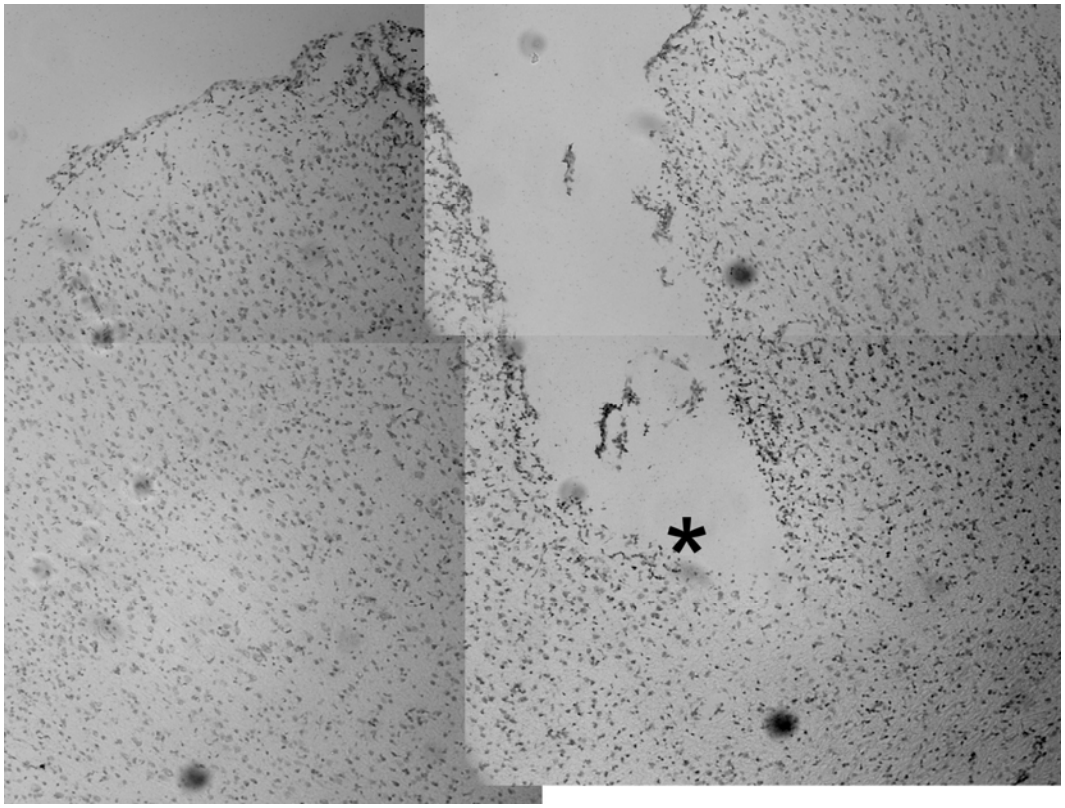

Figure 3.4: Left parietal cortex around an infusion site scar. Low power (10X) bright field composity map. Cresyl violet stain.

The overall intensity of fluorescent labeling remained more or less consistent for extended distances rostral and caudal to the FG injection site. The rostral-caudal distance between the first and last analyzed sections varied from animal to animal from 108 to 444 nm, and no quantitative differences were observed in the overall intensity of FG labeling between sections taken from any single animal regardless of the distance of that section from the FG injection site.

Some variability was also seen in the extent of the tissue damage associated with the mini-pump cannula implantation. In the majority of animals the entirety of the scar area could be seen encompassed within about half of a single low power (10X) field on light microscopy, the obvious scar could be seen on sequential sections through distances ranging from 100 to 200 nm apart. In most animals, some subtle evidence of tissue damage could usually be seen in all analyzed sections regardless of distance from implantation site. An example of a typical mini-pump cannula scar site is shown in figure 3.4.

Bright field microscopy also confirmed placement of mini-pump cannula into parietal cortex in all animals, as the distinct cytoarchitecture of parietal cortex with a thick, well defined granular layer IV was seen lateral to the cannula scar site in all animals, and medial to the scar site in most animals. Motor cortex could be identified histologically in some sections of some animals by the disappearance of an obvious granular layer IV in cortical areas some distance medial to the cannula scar site.

The completeness of the callosotomy was confirmed by histological examination under very low power (4X) bright field microscopy, showing a callosotomy scar transecting the entire thickness of the corpus callosum. The degree of tissue damage associated with the callosotomy scar ranged from mild to moderate. In two animals, attempted callosotomy failed to transect the corpus callosum at any point along the rostro-caudal extent of examined sections. In these animals the scar from the midline incision could be seen reaching the superior edge of the corpus callosum but not continuing further downwards to cut through the callosal fibers. These animals were subsequently analyzed as a separate sub-group. An example of the scar from a successful callosotomy is shown in figure 3.5.

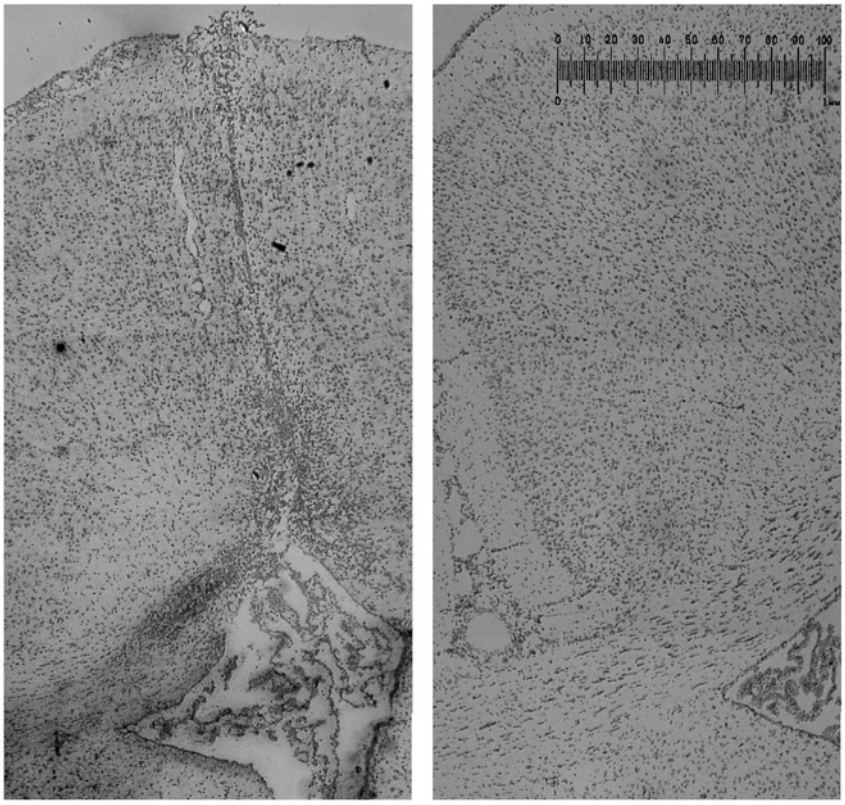

Figure 3.5: Callosotomy scar. Composite low-power (4X) micrographs. (Left panel) Callosotomy through the entirety of the corpus callosum into the underlying lateral ventricle. (Right panel) Comparison to a pristine corpus callosum.

High power composite micrographs of representative sections of experimental subgroups after in-situ hybridization are shown in figure 3.6. Qualitative differences can readily be seen between experimental groups. High densities of silver grains, representing elevated expression of GAP-43 mRNA, can be seen in the two experimental subgroups that had bilateral blockades of FGF-2 signaling: the bilateral antibody infusion subgroup (B) and the callosotomy with ipsilateral antibody infusion subgroup (E). In contrast, the control (A), ipsilateral antibody infusion (C), contralateral antibody infusion (D), and callosotomy with ipsilateral saline vehicle subgroups, which had either unilateral or no blockade of FGF-2 signaling, had lower densities of silver grains.

Interestingly, when fluorescent maps are overlaid on top of bright field images of the same area to identifiy transcallosal neurons, neurons not labeled by FG were also seen to have high densities of silver grain labeling. The numbers of such neurons were clearly highest in the two groups with bilateral FGF-2 signal blockade, but the occasional highly

labeled neuron could be seen in the unilateral FGF-2 blockade and control groups as well. These groups also had a few rare fluorescently labeled neurons exhibited high silver grain density, but the majority of observed neurons in these groups, both fluorescently labeled and not labeled, displayed only low silver grain density. In contrast, many neurons, regardless of fluorescent labeling, exhibited high silver grain density in the two bilateral FGF-2 signal blockade subgroups.

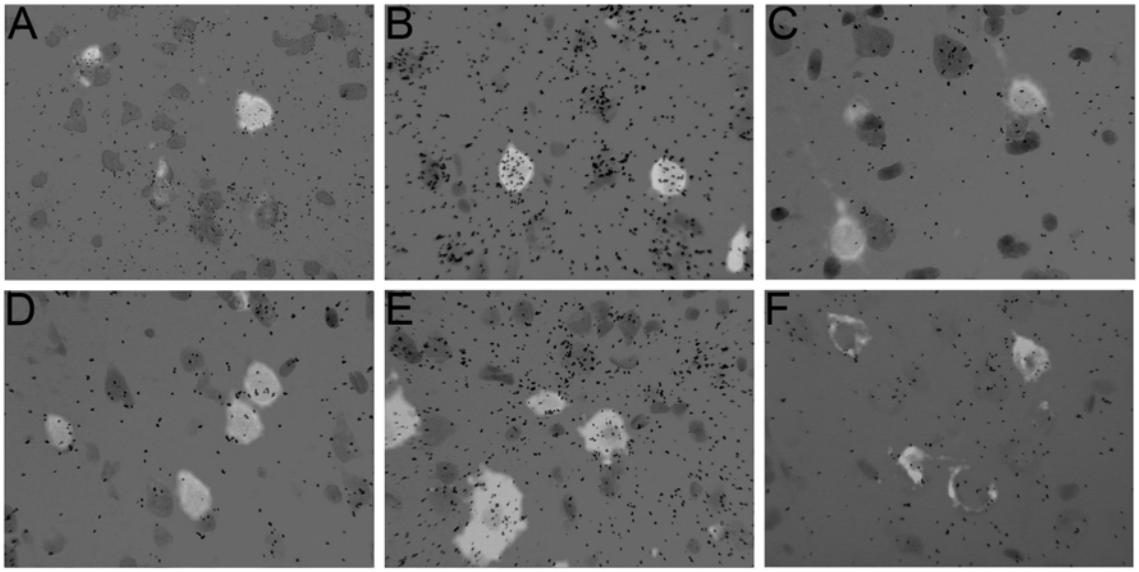

Figure 3.6: Representative composite micrographs of experimental groups. Overlay of fluorescent maps over bright field images identifies TCNs. Density of silver grains reveals GAP-43 mRNA expression within individual labeled cells. A) Control group (bilateral PBS). B) Bilateral antiFGF-2. C) Ipsilateral antiFGF-2. D) Contralateral antiFGF-2. E) Callosotomy with ipsilateral antiFGF-2. F) Callosotomy with ipsilateral PBS.

We chose not to investigate these non-fluorescently labeled neurons that appeared to up-regulate GAP-43 mRNA expression in this experimental project due to time and resource constraints. The identities and properties of these neurons is a potential area for fruitful future investigations.

3.2 SUMMARY OF RESULTS: MEAN LEVELS OF GAP-43 mRNA EXPRESSION

For those neurons that were successfully fluorescently labeled and thus identified as TCNs, the density of silver grain labeling was quantified for individual neurons. The mean levels of GAP-43 mRNA expression for all the labeled TCNs in each experimental sub-group were then calculated and are given in Table 3.1.

Table 3.1: Summary of Experimental Groups and Results

Experimental Group	N	n	GAP-43 mRNA Expression Mean ± Std Err	
Control Groups				
No intervention (FG only)	3	682	6.591 ± 1.160	
Bilateral Saline Vehicle (PBS)	3	931	5.725 ± 0.314	
Bilateral rabbit serum IgG*	3	801	6.466 ± 0.363	
Pooled Control	9	2414	6.261 ± 0.387	
				p-value**
Experimental Group 1				
Bilateral antiFGF-2***	5	2388	11.586 ± 0.449	0.000006
Ipsilateral antiFGF-2	3	740	7.558 ± 0.963	0.309
Contralateral antiFGF-2	3	1471	7.736 ± 0.607	0.113
Experimental Group 2				
Callosotomy + antiFGF-2	4	1645	12.723 ± 0.385	0.000001
Callosotomy + PBS	3	1250	6.782 ± 0.714	0.563
Incomplete Callosotomy + antiFGF-2	2	550	7.602 ± 0.109	0.021

N = number of animals in a group,
n = number of neurons analyzed in a group
* The concentration of rabbit serum IgG used was 9.0 ug/mL
** p-values calculated versus Pooled Control
*** The concentration of anti-FGF-2 for all experimental sub-groups using antiFGF-2 was 9.0 ug/mL

3.2.1 Control Group

Three separate experiments were designed to act as controls. In the first experiment, naïve animals treated only with FG injection to identify TCNs were analyzed to determine the baseline level of GAP-43 expression in adult rat parietal cortex. To ensure that nonspecific effects secondary to saline vehicle and/or cortical injury from mini-pump implantation itself would be accounted for, a second control experiment was performed with animals treated with bilateral infusions of saline vehicle alone (10 mM PBS). To determine what effects, if any, might be caused by non-specific effects of

immunoglobulins, a third control experiment was done using bilateral infusions of 9.0 ug/mL rat serum IgG.

Using a threshold value of 5 times background for labeled versus unlabeled neurons, the TCNs of each experimental animal were divided into four subpopulations based on their level of GAP-43 mRNA expression: no GAP-43 expression (<5 X background), low GAP-43 expression (5 to 10 X background), moderate GAP-43 mRNA expression (10 to 15 X background), and high GAP-43 mRNA expression (>15 X background). The mean proportions of neurons found in each subpopulation are plotted in figure 3.4 for each of the three control experimental groups for comparison.

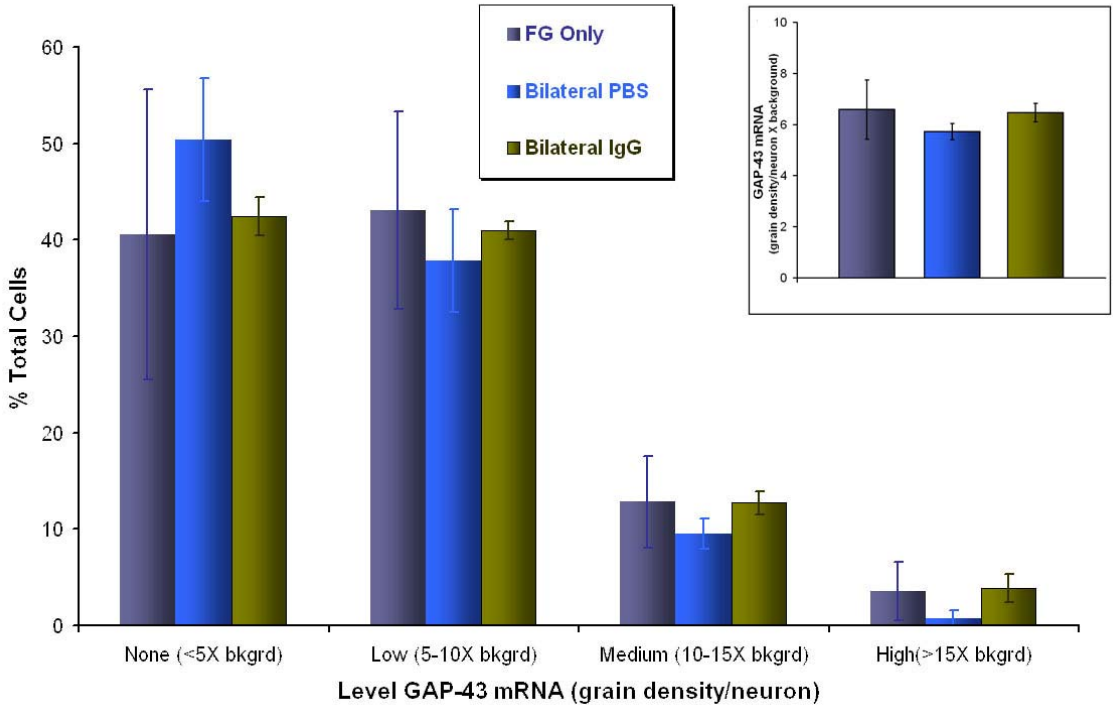

Figure 3.7: Proportion of neurons in control experiments with varying levels of GAP-43 mRNA expression. (Inset) Mean levels of GAP-43 mRNA expression in control experiments.

ANOVA analysis of the three control experimental sub-groups showed no significant differences between them for either mean GAP-43 mRNA expression levels ($F = 0.417$, $p = 0.677$) or the proportion of cells with high levels of GAP-43 mRNA expression. ($F = 0.200$, $p = 0.824$) Therefore, these results were combined together to

produce a single pooled group that was used as a control for comparison with all subsequent experimental sub-groups.

3.2.2 Experimental Group 1: Bilateral and Unilateral FGF-2 Blockade

In experimental group 1 we examined the levels of GAP-43 mRNA expression produced after 7 days of bilateral FGF-2 blockade using 9 ug/mL of anti-FGF-2 infused through osmotic mini-pumps. We hoped to block FGF-2 signaling from the majority of both local (ipsilateral) and distal (contralateral) axon target sites using this method, and therefore remove any retrograde repression that FGF-2 might be providing for the transcallosal neurons. We then compared the levels of GAP-43 mRNA expression obtained after bilateral FGF-2 blockade with that obtained after incomplete unilateral FGF-2 blockades.

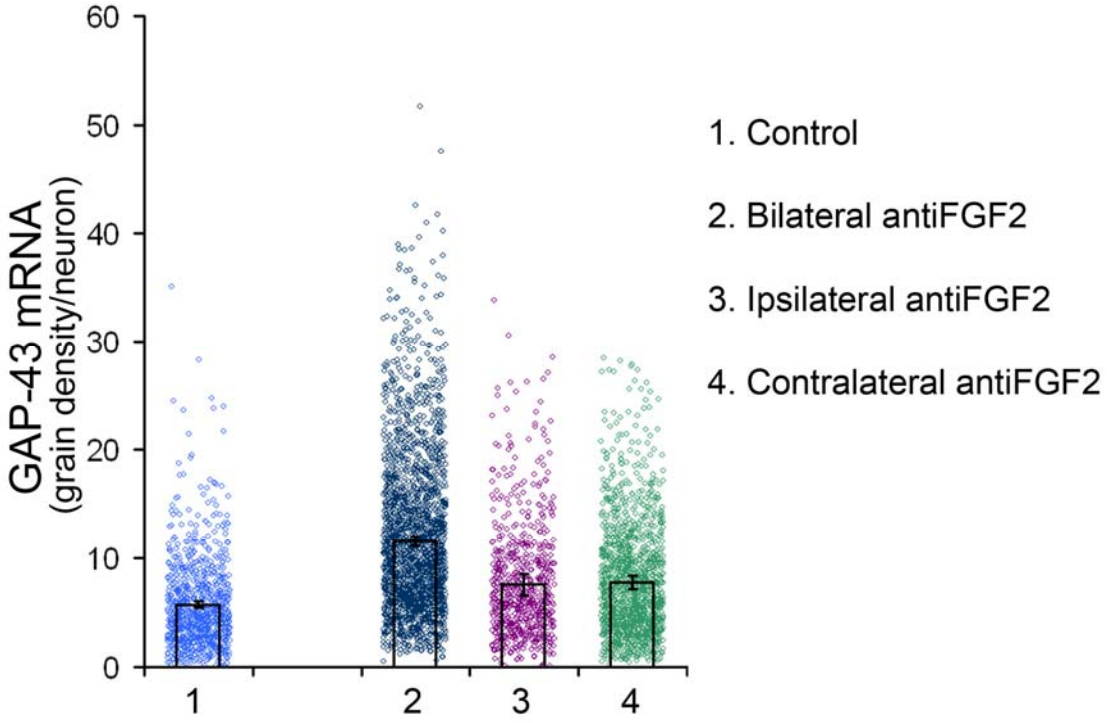

Figure 3.8: Results for experimental group 1, comparing bilateral, ipsilateral, and contralateral antibody infusion sub-groups with the pooled control group. GAP-43 mRNA levels for each individual TCN plotted, with mean values represented by overlaid bars. Error bars represent one standard error in either direction.

Theoretically, an ipsilateral anti-FGF-2 infusion should block FGF-2 signaling from local axon target sites, but not affect any signaling coming from the distal axon

target sites in the contralateral hemisphere. Similarly, a contralateral anti-FGF-2 infusion should block FGF-2 signaling from the distal axon target sites, but spare any signals provided by local axon target sites in the ipsilateral cortex.

The results for experimental group 1 are summarized in figure 3.8. Values for individual neurons are plotted as points while the mean level of GAP-43 mRNA expression calculated for controls and experimental group 1 are represented by the overlaid bar graph (numerical values given in table 3.1). Comparisons between individual sub-groups were conducted using Student's t-test. When the action of FGF-2 was blocked bilaterally at both local and distal axon target sites, the mean GAP-43 mRNA expression per TCN was significantly elevated when individually compared to the pooled control group value ($p = 0.000006$), the ipsilateral antibody infusion subgroup ($p = 0.034$), and the contralateral antibody infusion subgroup ($p = 0.006$). ANOVA analysis of the bilateral, ipsilateral and contralateral subgroups together also showed a significant difference between them ($F = 14.35$, $p = 0.002$). In contrast, the mean GAP-43 mRNA expression per TCN observed in animals that received ipsilateral or contralateral antibody infusions where not significantly different from the pooled control, ($p = 0.309$ and $p = 0.113$ respectively) or each other. ($p = 0.88$) Thus bilateral FGF-2 blockade resulted in an increase of GAP-43 expression in TCNs while unilateral blockades in isolation, both ipsilateral and contralateral, had no significant effect.

3.2.3 Experimental Group 2: Callosotomy With and Without Local FGF-2 Blockade

Having found that a bilateral blockade of FGF-2 signaling appeared to result in an up-regulation of GAP-43 expression, we next wanted to investigate the regulation of GAP-43 expression after axonal injury in transcallosal neurons. Since all contralateral target signals to TCNs should in theory be interrupted by callosotomy, the bilateral infusion results suggested that a local ipsilateral blockade of FGF-2 signaling should result in an up-regulation of GAP-43 mRNA expression when combined with callosotomy. Therefore, in experimental group 2, we investigated the effect of stereotactic callosotomy (see figure 3.5) on the expression of GAP-43 in transcallosal neurons 7 days post axotomy, with or without a simultaneous blockage of local FGF-2 signaling by ipsilateral antibody infusion via an osmotic mini-pump.

The results for this experimental group are summarized in figure 3.9 (numerical values given in table 3.1). Callosotomized animals treated with simultaneous ipsilateral antibody infusion had mean levels of GAP-43 mRNA significantly higher than the control group. (p=0.000001) On the other hand, callosotomized animals that received ipsilateral infusions of only saline vehicle had a mean GAP-43 mRNA level which was not significantly different from the control group. (p = 0.563)

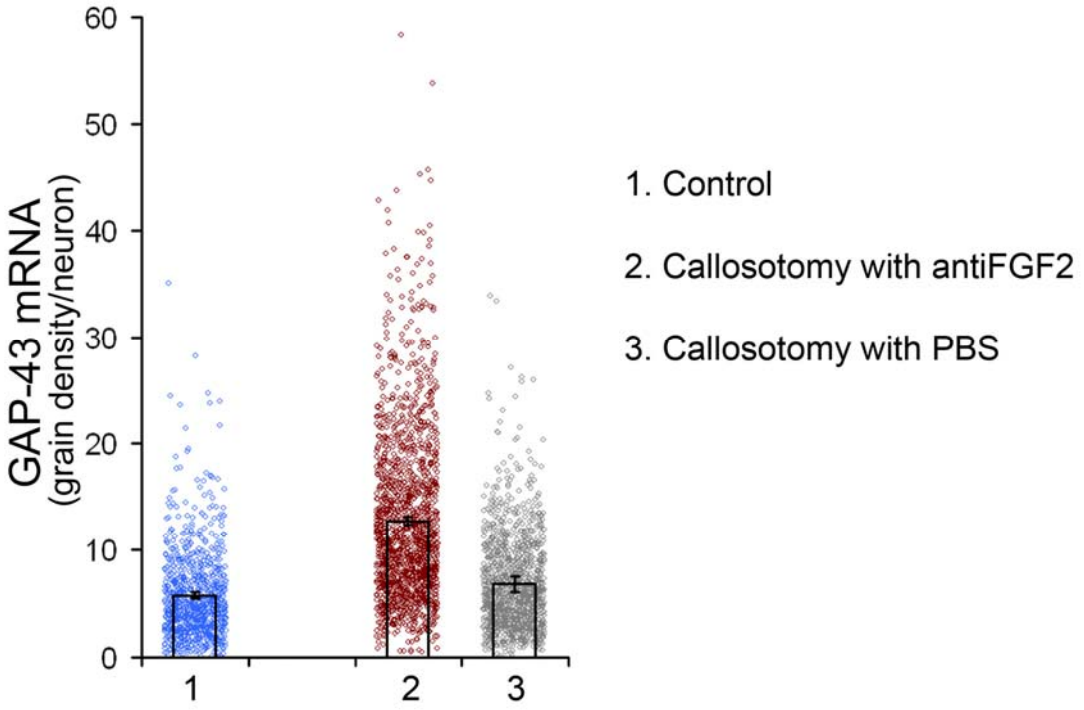

Figure 3.9: Results for experimental group 2, comparing callosotomy with ipsilateral antibody infusion and callosotomy without antibody infusion sub-groups with the control group. Mean GAP-43 mRNA levels represented by bars overlaid on top of dot plots on each individual neuron. Error bars represent one standard error in either direction.

In the two animals where the callosotomy procedure had failed, and the corpus callosum was not completely transected, the GAP-43 mRNA levels were substantially lower than the levels observed in the completely callosotomized subgroup, similar to the levels observed in the ipsilateral and contralateral antibody infusion subgroups in experimental group 1, and not much higher than that seen in the fully callosotomized animals that did not receive the antibody infusion. When compared with the pooled

control group, the small increase in GAP-43 expression reached significance ($p = 0.021$), but given an n of only 2, this calculation is inconclusive.

3.2.4 Between Group Comparisons

Comparing experimental groups 1 and 2 together, no significant differences in GAP-43 expression were seen between the bilateral antibody infusion group and the callosotomy with ipsilateral antibody infusion. ($p = 0.16$) ANOVA analysis of all six sub-groups together indicated significant difference. ($p < 0.0001$) ANOVA analysis of the control group, both unilateral infusion groups, and the callosotomy group without antibody infusion showed no significant differences between these groups when analyzed together. ($p = 0.27$)

These results show that a bilateral blockade of FGF-2 signaling is sufficient to cause up-regulation of mean levels GAP-43 mRNA in transcallosal neurons. We found no significant difference regardless of how the blockade was actually achieved, either by blocking FGF-2 biochemically at the location of axon target sites, or physically interrupting the axon tracts. We also did not see any significant elevation of GAP-43 expression resulting from any experimental manipulation that resulted in only a partial blockade of either the ipsilateral or the contralateral axon targets of TCNs.

3.3 SUBPOPULATION ANALYSIS

3.3.1 Neuron Subpopulations with Different Levels of GAP-43 Expression

A wide range of levels of GAP-43 mRNA was observed in individual TCNs, as demonstrated by the wide scatter in individual plot points seen in figures 3.8 and 3.9, in all experimental subgroups. Regardless of the experimental manipulation, in any given animal, transcallosal neurons were found to exhibit levels of GAP-43 expression that varied from low to high. While the number of neurons with high levels of GAP-43 expression was clearly greater in the bilateral antibody infusion and the callosotomy with antibody infusion groups compared to the others, both these sub-groups nevertheless still had many neurons that remained with lower levels of GAP-43 expression. Conversely,

even in the control group, a few neurons with higher levels of GAP-43 expression were observed.

Individual neurons were arbitrarily sorted into subpopulations with no, low, medium, and high levels of GAP-43 mRNA expression for the subgroups in experimental groups 1 and 2 in the same way as with the three control subgroups, as described in section 3.1.1, and compared with the pooled control group. This is illustrated in figure 3.10. The numerical values for the percentage of neurons in each of the subpopulations with varying levels of GAP-43 expression are shown in table 3.2. Large subpopulations of TCNs with medium and high levels of GAP-43 mRNA were seen only in the bilateral antibody infusion and the callosotomy with antibody infusion sub-groups.

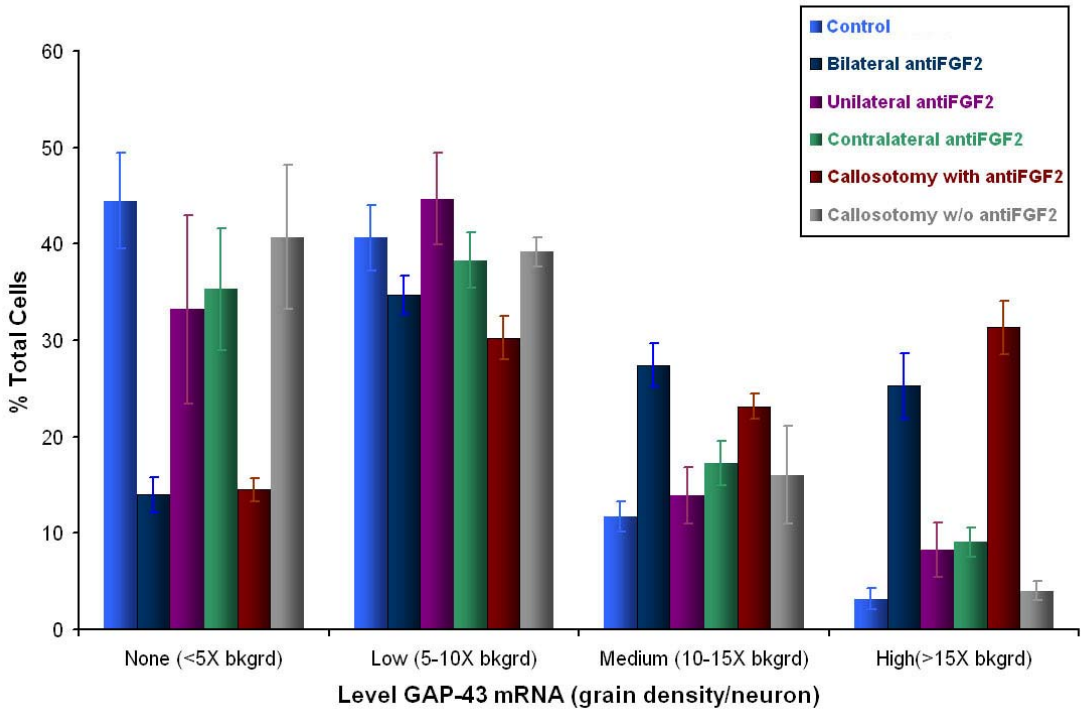

Figure 3.10: Proportions of neurons with varying levels of GAP-43 expression in each experimental sub-group.

Statistical analysis of subpopulation proportions confirmed the results found with whole population mean levels of GAP-43 mRNA. The proportion of neurons with high levels of GAP-43 expression were significantly higher in both the bilateral antibody infusion and callosotomy with antibody infusion sub-groups when compared with the pooled control group ($p = 0.0005$ and $p = 0.003$, respectively) and not significantly

different from each other. ($p = 0.15$) ANOVA analysis once again showed that each of these two sub-groups were significantly higher than all the others.

Table 3.2: Proportion of neurons with low, moderate, and high levels of GAP-43 Expression in the Experimental Sub-Groups

Experimental Group	Percentage of neurons per subpopulation (± Standard Error)			
	None (<5X)	Low (5-10X)	Moderate (10-15X)	High (>15X)
Pooled Control	44 ± 5.0	41 ± 3.4	12 ± 1.6	3.2 ± 1.1
Experimental Group 1				
Bilateral antiFGF-2*	12 ± 2.2	37 ± 2.5	27 ± 1.7	25 ± 2.7
Ipsilateral antiFGF-2	33 ± 9.8	45 ± 4.8	14 ± 2.9	8.3 ± 2.8
Contralateral antiFGF-2	35 ± 6.3	38 ± 2.9	17 ± 2.3	9.1 ± 1.5
Experimental Group 2				
Callosotomy + antiFGF-2	14 ± 1.2	30 ± 2.2	23 ± 1.3	32 ± 2.3
Callosotomy + PBS	41 ± 7.5	39 ± 1.5	16 ± 5.1	4.0 ± 1.0

* The concentration of antiFGF-2 for all experimental sub-groups using antiFGF-2 was 9.0 ug/mL

Similarly, the bilateral antibody and callosotomy with ipsilateral antibody sub-groups also had a significantly lower proportion of neurons no GAP-43 mRNA expression ($p = 0.0001$ and $p = 0.0002$ respectively) and not significantly different from each other ($p = 0.65$).

Visual inspection of the dot plots in figures 3.8 and 3.9 did not reveal any obvious clustering of neurons into groups with high or low levels of GAP-43 expression. In order to determine whether or not there was discreet clustering of GAP-43 expression in individual TCNs into distinct ranges of values, the individual neurons in each experimental subgroup were pooled and sorted into 1 unit wide bins in order to construct population histograms, which are shown in figures 3.11 and 3.12.

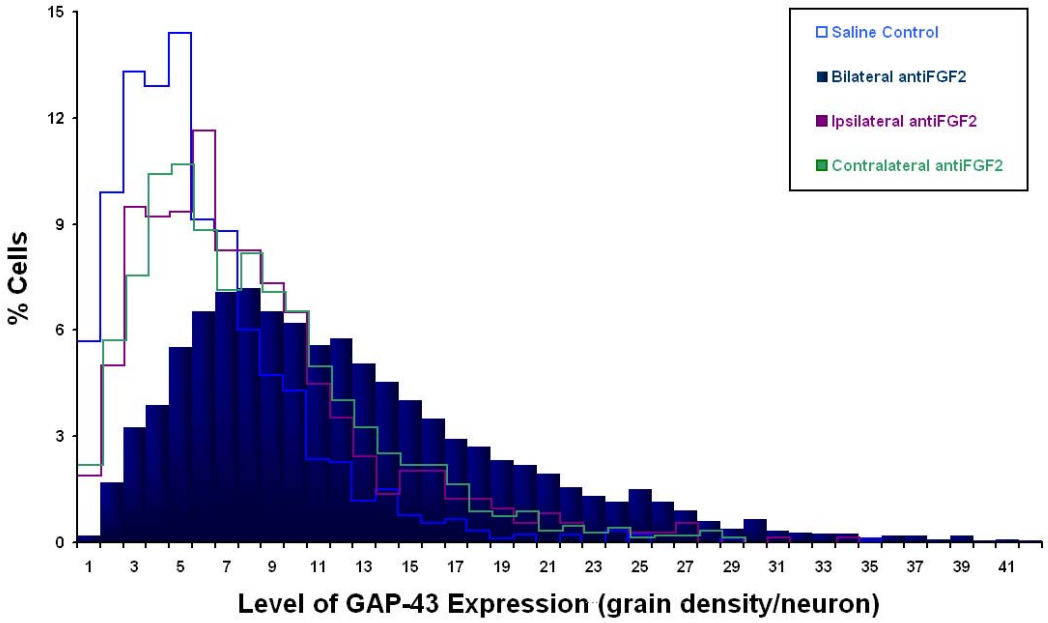

Figure 3.11: Population histograms for experimental group 1, showing proportions of cells with varying levels of GAP-43 mRNA expression compared with the control group.

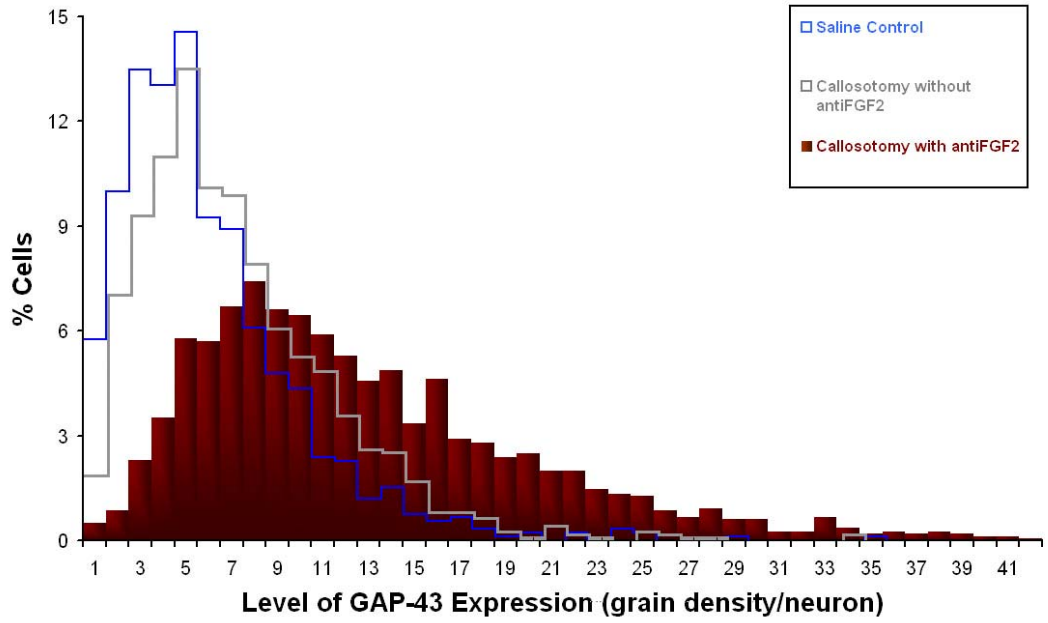

Figure 3.12: Population histograms for experimental group 2, showing proportions of cells with varying levels of GAP-43 expression compared with the control group.

An apparent unimodal distribution of GAP-43 expression in all experimental sub-groups is seen, without distinct peaks. The distribution of neurons is shifted rightward in the bilateral antibody infusion and the callosotomy with ipsilateral antibody subgroups, with greater proportions of neurons expressing higher levels of GAP-43 mRNA.

3.3.2 Correlations of GAP-43 Expression with Cross-sectional Neuron Area

Because transcallosal neurons are generally of similar size and shape, the cross-sectional area of any given neuron in a particular section would relate to the plane of transection through that particular neuron. Comparisons of GAP-43 expression between sectioned neurons may be confounded if the distribution of GAP-43 mRNA in TCNs were uneven. For example, if GAP-43 mRNA in TCNs clustered predominantly at the poles of the cell, then neurons with smaller cross-sectional areas may have preferentially higher levels of measured GAP-43 expression compared to neurons with larger cross-sectional areas, since cross sections through the center of a pyramidal neuron would tend to be larger than cross sections through a pole.

To ensure that the measurements of GAP-43 expression in TCNs was not affected by this possibility, the neuronal cross-sectional area of each analyzed TCN was obtained using a Northern Eclipse subroutine, and correlated with observed levels of elevated GAP-43 expression in the bilateral antibody infusion and the callosotomy with antibody infusion sub-groups. The mean neuron cross-sectional area was calculated for each of the no label, low, moderate, and high levels of GAP-43 mRNA expression subpopulations, and compared. These results are given in table 3.3. No differences were found in the mean neuron cross sectional area between any of the subpopulations with differing levels of GAP-43 expression.

The absence of differences in cross sectional areas between neurons with differing levels of GAP-43 expression also suggests that there were no major sampling differences between different sections taken from different animals, and that the experimental procedures did not differentially cause atrophy or hypertrophy in TCNs.

Table 3.3: Mean size and distance to injection site for neurons expressing no, low, moderate, and high levels of GAP-43 mRNA

Experimental Sub-Group	Neuronal Subpopulation			
	Unlabeled (<5X)	Low (5-10X)	Moderate (10-15X)	High (>15X)
Cell Cross Sectional Area (nm^2)				
Bilateral antiFGF-2*	133.9 ± 8.2	135.0 ± 7.3	134.9 ± 5.1	133.7 ± 10.6
Callosotomy + anti-FGF-2	135.5 ± 5.6	135.5 ± 10.1	137.5 ± 11.8	142.9 ± 16.0
Distance to injection site (μm)				
Bilateral antiFGF-2	136.1 ± 5.4	139.4 ± 9.0	136.4 ± 12.8	135.6 ± 12.2
Callosotomy + antiFGF-2	124.6 ± 30.5	140.8 ± 22.3	137.9 ± 19.0	130.2 ± 15.7

* the concentration of antiFGF-2 for all experimental sub-groups using antiFGF-2 was 9.0 ug/mL

3.3.3 Correlations of GAP-43 mRNA Expression with the Distance of the Cell Body to the Injection Site

One potential explanation for the distribution of varying levels of GAP-43 expression observed is that it was a result of a concentration gradient of the antibody diffusing out from the ipsilateral mini-pump infusion site. If this were indeed true, it would be expected that neurons situated closer to the injection site should, on average, have higher levels of GAP-43 mRNA expression than neurons further away.

In order to investigate this possibility, the center of the injection site was determine to be located on the section that was exact halfway between the first section where the injection site scar became visible and the last section on which the scar was visible. The distance of each analyzed section from the injection site section was calculated based on the number of cuts separating the two sections and the width of each individual section. On each analyzed section, the distance of each labeled transcallosal neuron to the injection site scar was directly measured. The actual distance between the

neuron and the center of the injection site was thus calculated using the following formula:

$D = SQRT (d_a^2+d_b^2)$

Where:

D = distance between neuron and center of injection site

d_a^2 = measured distance between neuron and injection site scar on tissue section

d_b^2 = calculated distance between tissue section and section with center of injection site

The mean distances for each of the unlabeled, low, moderate, and high GAP-43 expression subpopulations were calculated for the bilateral antibody infusion and callosotomy with antibody infusion sub-groups, and given in table 3.3. Once again, no discernable differences were observed between neurons far away and closer to the injection site.

3.3.4 Levels of GAP-43 Expression in Medial versus Lateral Neurons

If the antibody diffused widely throughout the brain, then the possibility also exists that the antibody may have diffused across the midline, and both the ipsilateral and contralateral axon terminals of some transcallosal neurons may have been affected by antibody originating from a single site. If this did happen, the effect should be most marked in the bilateral infusion experimental sub-group, where antibody diffusing across the midline from both sides would have created a zone of additively high concentration of diffusing antibody medial to the infusion sites. One might then expect that neurons whose cell bodies were situated relatively medial might have higher levels of GAP-43 expression, with their distal axon collaterals projecting to the homologous area of medial contralateral cortex.

Also, in the animals used in these experiments, primary motor cortex lay medial to the infusion site, while somatosensory cortex lay lateral. While most of the neurons analyzed were clearly within sensory cortex, some of the most medial neurons may have been in motor cortex, and potential differences in GAP-43 expression between TCNs in sensory and motor cortex may have had an effect.

The mean levels of GAP-43 expression found in transcallosal neurons with cell bodies medial to or lateral to the ipsilateral infusion site scar were compared for the bilateral antibody infusion and the callosotomy with contralateral infusion subgroups. In the bilateral antibody infusion sub-group, the mean GAP-43 expression in medial neurons was 12.26 +/- 0.79, compared to 10.05 +/- 0.62 for lateral neurons ($p = 0.09$). While not statistically significant, a trend towards medial neurons having higher GAP-43 expression levels in response to bilateral FGF-2 blockade is observed.

In the callosotomy with antibody infusion group, it would not be expected to observe any medial to lateral variation in GAP-43 expression, because the callosotomy would theoretically obliterate all signals originating from the contralateral side, such that the presence of contralateral antibody should not matter. No significant differences were observed in this experimental sub-group between medial and lateral neurons, irrespective of their laminar location, (13.01 +/- 0.86 and 11.95 +/- 0.93, $p = 0.28$ respectively) but the trend towards medial neurons having higher GAP-43 expression levels still remains, although it is statistically less strong. These results suggest the possibility of a small effect from overlapping diffusion crossing the midline from the two bilateral infusion sites, combined with the presence of a small population of TCNs in motor cortex that respond to bilateral FGF-2 blockade with higher GAP-43 upregulation than parietal TCNs. Thus the GAP-43 expression levels from medial neurons would be skewed upwards by a combination of both effects in the bilateral antibody infusion subgroup, but only by the motor neuron effect in the callosotomy with ipsilateral antibody infusion subgroup.

3.3.5 Laminar Location of Transcallosal Neurons and Levels of GAP-43 Expression

In rodents, transcallosal neurons are found relatively evenly distributed between cortical layers II/III and V. (Conti and Manzoni, 1994; Swanson, 2003) The assembly of the topographic organization of the neocortex is time dependent in development, such that neurons residing in different layers are born at different times.(Bielas et al., 2004) It is therefore possible that the transcallosal neurons in layer II/III and those in layer V may consist of distinct populations of neurons with differing properties.

Individual TCNs in the control, bilateral antibody infusion, callosotomy without antibody infusion, and callosotomy with ipsilateral antibody infusion subgroups were sorted by visual inspection according to their position relative to cortical layer IV. All supragranular TCNs were considered to be in layer II/III while infragranular neurons were classified as belonging to layer V. For a small number of neurons that appeared to be in medial motor cortex where cortical layer IV was indistinct, the dividing line was arbitrarily estimated using the level of the layer III/IV junction in the neighbouring lateral parietal cortex. The mean levels of GAP-43 expression were determined for layer II/III and layer V neurons and compared to see if there were any significant differences. These results are shown in table 3.4 and figure 3.13.

Table 3.4: Differences in levels of GAP-43 expression observed in transcallosal neurons in cortical layer II/III and V

Experimental Sub-group	Proportion Layer V(%)	Mean GAP-43 Expression		Magnitude increase (X control)	
Layers		II/III	V	II/III	V
Control	43.7 ± 3.0	5.44 ± 0.37	7.21 ± 0.63		
Bilateral antiFGF-2*	49.2 ± 6.2	10.20 ± 0.48	13.19 ± 0.69	1.88	1.83
Callosotomy with antiFGF-2	47.0 ± 4.6	10.76 ± 0.41	15.35 ± 0.74	1.98	2.13
Callosotomy with PBS	38.4 ± 0.5	5.95 ± 0.53	8.12 ± 0.99	1.09	1.13

* the concentration of antiFGF-2 in all experimental sub-groups using antiFGF-2 was 9.0 ug/mL

Statistically significant differences were found between the mean levels of GAP-43 expression in layer II/III compared to layer V in the bilateral antibody infusion and callosotomy with antibody infusion experimental sub-groups ($p = 0.009$ and $p = 0.01$ respectively). A significant difference between layers was also found in the pooled control group ($p = 0.03$). No significant difference in levels of GAP-43 expression was found between layers II/III and layer V in the callosotomy with saline vehicle experimental sub-groups ($p = 0.15$) although there was a trend towards higher expression

in layer V.

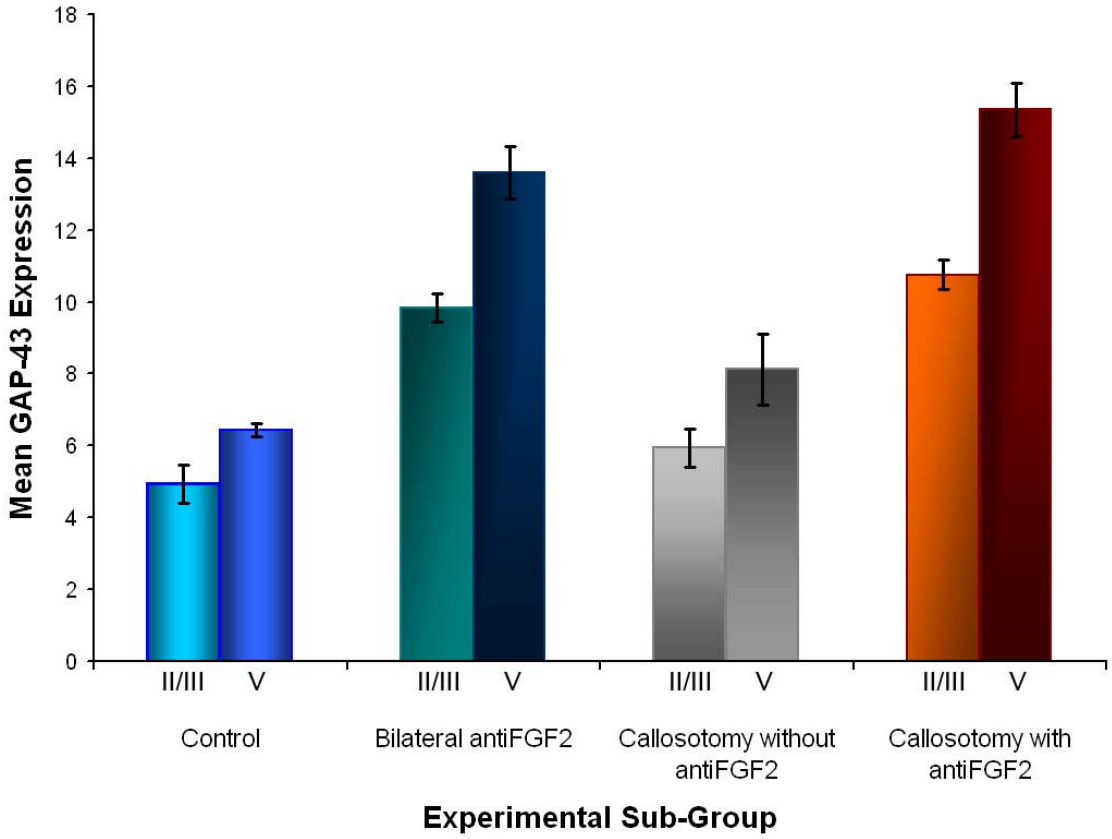

Figure 3.13: Comparison of mean GAP-43 expression between cortical layers II/III versus V.

When the labeled transcallosal neurons in their separate layers are sorted into subpopulations with no, low, moderate, and high levels of GAP-43 expression, in layer II/III, 15.7 +/- 2.9 % were found to be unlabeled, 41.5 +/- 1.6 % had low levels of GAP-43 expression, 25.8 +/- 1.3 % had moderate levels of GAP-43 expression, and 17.0 +/- 2.4 % had high levels of GAP-43 expression. In layer V, 9.6 +/- 1.3 % of the transcallosal neurons were unlabeled, 29.7 +/- 5.0% had low levels of expression, 27.2 +/- 1.5% had moderate levels of expression, and 33.4 +/- 4.0% had high levels of expression. The comparison in the proportion of neurons with varying levels of GAP-43 expression between layers II/III and V is illustrated in figure 3.14

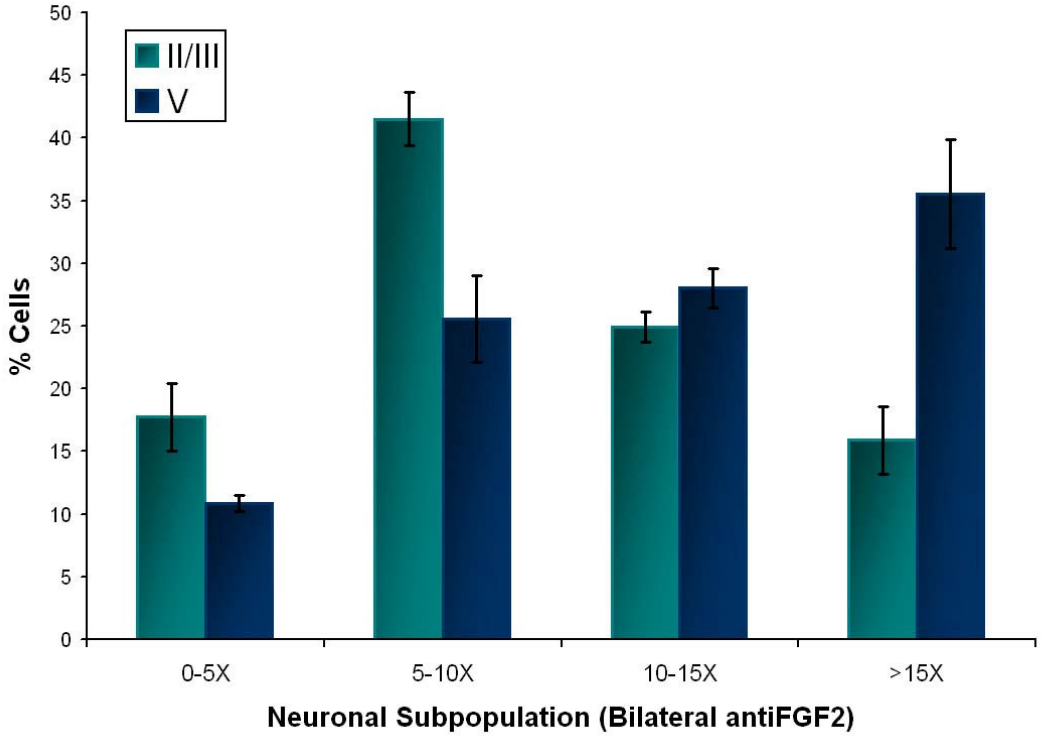

Figure 3.14: Comparison of the proportion of TCNs with different levels of GAP-43 expression between cortical layers II/III versus V for the bilateral antibody infusion experimental sub-group.

A very similar pattern of distribution comparing layer II/III and layer V was found in the callosotomy with antibody infusion experimental sub-group. Here, in layer II/III, 17.7 +/- 1.4 % of neurons were unlabeled, 37.5 +/- 3.0 % had low levels, 20.7 +/- 3.1 % had moderate levels, and 24.1 +/- 4.5 % had high levels of GAP-43 expression, while in layer V, 9.3 +/- 2.6 % were unlabeled, 22.4 +/- 2.8 % had low levels, 25.1 +/- 0.4 % had moderate levels, and 43.2 +/- 2.5% had high levels of GAP-43 expression. The comparison between the two layers for this experimental sub-group is illustrated in figure 3.15.

In both the bilateral antibody infusion and the callosotomy with antibody infusion experimental sub-groups, the highest proportion of layer II/III transcallosal neurons had low levels of GAP-43 expression, while in layer V, the highest proportion of neurons had high levels of GAP-43 expression. Significantly more neurons had high levels of GAP-43 expression in layer V than in layer II/III in both experimental sub-groups ($p = 0.01$ and $p = 0.03$ respectively).

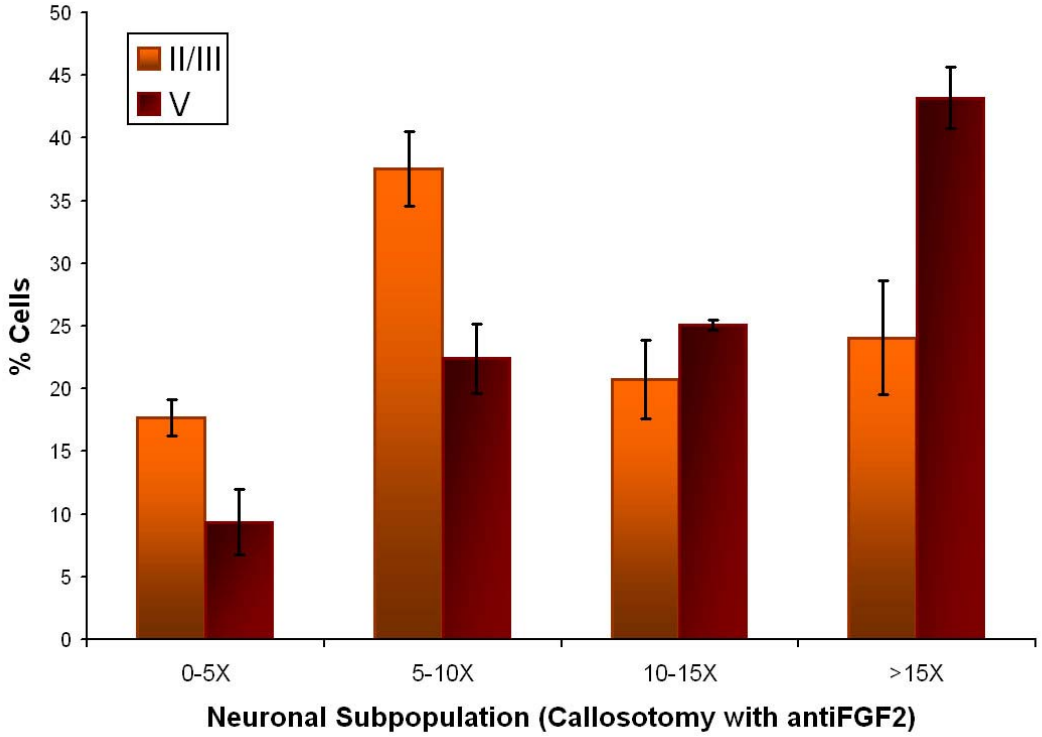

Figure 3.15: Comparison of the proportion of TCNs with different levels of GAP-43 expression between cortical layers II/III compared versus V for the callosotomy with antibody infusion experimental sub-group.

The callosotomy with antibody infusion sub-group also had significantly more neurons with low levels of GAP-43 expression in layer II/III than layer V, ($p = 0.02$), while there was a trend to more neurons with low levels of GAP-43 in layer II/III in the bilateral antibody infusion group. ($p = 0.07$) There was a trend towards a higher proportion of unlabeled neurons in layer II/III compared to V, ($p = 0.08$ and $p = 0.06$) while there was no significant difference between layers in the proportion of neurons that had moderate levels of GAP-43 expression ($p = 0.17$ and $p = 0.30$). When the bilateral antibody infusion and callosotomy with antibody infusion experimental sub-groups are compared with each other, there were no significant differences between them in the proportion of neurons with no, low, or moderate levels of GAP-43 expression, and a trend towards a higher proportion of neurons with high levels of GAP-43 in the callosotomy with antibody infusion sub-group. (For unlabeled neurons, $p = 0.56$ for layer II/III and $p = 0.92$ for layer V; for neurons with low levels of expression, $p = 0.32$ for layer II/III and $p = 0.25$ for layer V; for neurons with moderate levels of expression, $p =$

0.24 for layer II/III and p = 0.22 for layer V; for neurons with high levels of expression, p = 0.26 for layer II/III and p = 0.08 for layer V).

In both the bilateral antibody infusion and callosotomy with antibody infusion experimental sub-groups, the proportion of transcallosal neurons that are found in layer V was found to increase as the level of GAP-43 expression increases. This is illustrated in figure 3.16. In the bilateral antibody infusion sub-group, the proportion of layer V neurons was 39.8 +/- 6.2 % in unlabeled neurons, 42.0 +/- 8.6 % in neurons with low levels of expression, 51.3 +/- 6.1 % in neurons with moderate levels of expression, and 66.0 +/- 3.2 % in neurons with high levels of expression. A trend towards a higher proportion of layer V neurons was found between neurons with high and low levels of GAP-43 expression, (p = 0.05) and the proportion of layer V neurons was significantly higher in neurons with high levels of GAP-43 expression when compared to unlabeled neurons. (p = 0.01)

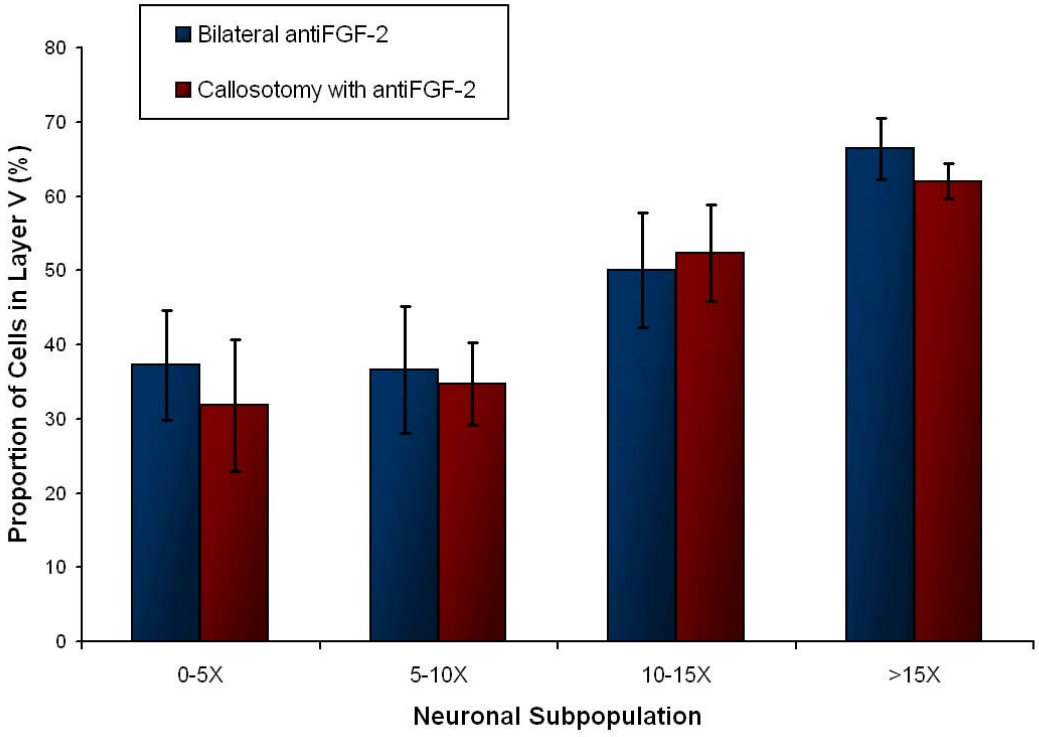

Figure 3.16: The proportion of transcallosal neurons in cortical layer V in neuronal subpopulations with varying levels of GAP-43 expression.

In the callosotomy with antibody infusion sub-group, the proportion of layer V neurons was 31.8 +/- 8.9 % in unlabeled neurons, 34.7 +/- 5.6 % in neurons with low expression, 52.3 +/- 6.5 % in neurons with moderate expression, and 62.0 +/- 2.3 % in neurons with high expression. A significant difference in the proportion of layer V neurons were found between neurons with high and neurons with low levels of GAP-43 expression, ($p = 0.03$) and a trend towards a higher proportion of layer V neurons was found when comparing neurons with high levels of GAP-43 expression with unlabeled neurons. ($p = 0.07$)

Thus, it appears that TCNs in layer V have a higher level of GAP-43 expression than TCNs in layer II/III. After bilateral interruption of FGF-2 signaling, a significantly higher proportion of layer V neurons display high levels of GAP-43 expression, while a plurality of layer II/III neurons show only low levels of GAP-43 expression. Also, the proportion of neurons that are in layer V is higher among neurons with high levels of GAP-43 expression after bilateral FGF-2 blockade than among neurons with lower levels of GAP-43 expression. These observed differences may be merely due to a greater shift towards higher levels of GAP-43 expression in layer V neurons, resulting in greater relative numbers in the highest of arbitrarily determined groups, but may also suggest the possibility that bilateral FGF-2 blockade negatively affects the survival of layer II/III neurons relative to layer V neurons.

For both layer II/III and layer V, however, the magnitude of the increase over the control group was similar. (1.88 versus 1.83 times control in the bilateral infusion subgroup, and 1.98 versus 2.13 times control in the callosotomy and ipsilateral infusion subgroup.) Because a significant difference between mean GAP-43 expression already existed in the pooled control group, the observed differences in the proportions of neurons with high and low levels of GAP-43 expression between layers II/III and V in the experimental subgroups may not represent any real difference in the response of these neurons to bilateral FGF-2 blockade, but simply equivalent magnifications of pre-existing differences in constitutive GAP-43 expression.

DISCUSSION AND CONCLUSIONS

4.0 FGF-2 AS A RETROGRADE REPRESSOR OF GAP EXPRESSION IN TRANSCALLOSAL NEURONS

Our results provide evidence that GAP expression in at least one population of neurons in the mature central nervous system is subject to regulation by the mechanism of retrograde repression, which had previously been postulated to operate in the peripheral (Bisby, 1988; Schreyer and Skene, 1993) developing central nervous system. (Karimi-Abdolrezaee and Schreyer, 2002). We have found evidence that in parietal transcallosal neurons, one of the substances involved in the retrograde repression signal in vivo is FGF-2. At least some subpopulations of transcallosal neurons up-regulate GAP-43 expression when FGF-2 signaling is blocked.

Our results are consistent with in vivo experiments that show that FGF-2 inhibits process outgrowth in cultured transcallosal neurons, (Catapano et al., 2004) and stand in contrast to observations of the actions of FGF-2 observed in amphibian retinal ganglia neurons, where FGF-2 has been shown to promote GAP expression after injury. (Soto et al., 2003)

Evolutionary differences between amphibians and mammals may partly account for some of these observations. After all, mature amphibian CNS neurons are known to possess a greater capacity for axon regeneration than their mammalian CNS counterparts. FGF-2 may well act as a promoter of GAP-43 expression in amphibian retinal ganglion cells, but as a retrograde repressor in mammalian transcallosal neurons. Also, in the Soto experiments, FGF-2 was exogenously applied directly to the transected optic nerve stumps as a single 5 minute pulse immediately after the injury (Soto et al., 2003), while in our experiments, the activity of endogenous FGF-2 was blocked continuously over the entire course of the experiment. These differences in technique may have contributed to

the different results. For example, FGF-2 may be present endogenously at only very low levels, and act as a retrograde repressor in low concentrations, but may have an opposing activity when applied exogenously in high concentrations.

It is not necessarily surprising that the activity of any given signaling agent in one given neuron population would turn out to be different in a different neuron population. In fact, differential responses among different axon populations should be expected. Axon tracts converge and diverge throughout the CNS and are often precisely targeted to sites in close proximity to one another. It makes perfect sense for a signaling molecule to promote elongation for one population of axons at a given time and place, while simultaneously repressing growth in another axon population, or even change from promotion to repression or vice versa for the same axon population at different times. In this regard it is likely that FGF-2 may be acting as an initiator of a wide range of downstream effects acting differently on different populations of neurons and glial cells in the central nervous system.

4.1 BILATERAL BLOCKADE OF FGF-2 IS NECESSARY FOR UP-REGULATION OF GAP-43 EXPRESSION TO OCCUR

A significant elevation of GAP-43 levels was observed only after a bilateral blockade of FGF-2 signaling. It was necessary for both ipsilateral and contralateral FGF-2 signaling to be blocked. No significant differences were observed between pharmacologic contralateral blockade with antibody infusion versus physical contralateral blockade with callosotomy. The only thing that mattered was whether or not the ipsilateral action of FGF-2 was also simultaneously blocked.

This supports our hypothesis that the presence of sustaining collaterals acts to maintain retrograde repression in the CNS and prevent GAP up-regulation after injury. GAP-43 expression did not increase in all experimental sub-groups with unilateral FGF-2 blockades, whether pharmacologic or physical, ipsilateral or contralateral. Retrograde repression appeared to remain active so long as FGF-2 signaling remained intact at the axon targets of at least one hemisphere, and no significant elevation of GAP-43 expression was observed.

The results from the experimental sub-group that had incomplete callosotomies supports the idea that actual interruption of retrograde signaling was required to substantially up-regulate GAP-43 expression. Reactive scarring and other injury effects in the midline cortex and glia surrounding the corpus callosum was not sufficient, if the majority of axons remained physically in continuity and no contralateral antibody infusion was used to block FGF-2 action at the target sites. Also supporting this hypothesis is the observation that ipsilateral anti-FGF-2 infusion alone does not result in significant up-regulation of GAP-43. This indicates that the FGF-2 does not act directly on the transcallosal neuron cell body, but instead is active at axon target sites, and the signal is carried to the neuron cell body by retrograde axonal transport.

No positional effects suggestive of a concentration gradient of antibody were observed. The location of the TCN cell body in relation to either the infusion site or the midline did not have any noticeable impact on GAP-43 expression. Several factors may account for this observation. Firstly, the antibody may have diffused far enough within the brain that concentration differences between labeled neurons closer and further from the injection site became insignificant. Secondly, there may have been a threshold concentration needed for the antibody to block FGF-2 signaling in affected neurons, above which no further effects would be observed, and this threshold concentration was reached for the majority of the neurons sensitive to FGF-2 blockade that were analyzed. Thirdly, these results may have been confounded by the fact that it is the concentration of antibody found at the axon target sites, and not the cell body, that would be most important in blocking retrograde repression, and the terminal axon arborizations of the analyzed transcallosal neurons may have been sufficiently spread out within the area through which the antibody diffused that the actual location of the neuron cell body did not matter.

The lack of observable positional effect also reduces the possibility that limitations to precision inherent in the surgical technique had any significant impact on the results. Variations in the precise implantation sites on ipsilateral and contralateral sides relative to each other, and between implantation sites from experimental animal to experimental animal might have been expected to result in individual transcallosal neurons being exposed to differing concentrations of antiFGF-2 at their contralateral and

ipsilateral axon target sites. This imprecision may therefore have contributed to some of the observed differences in GAP-43 expression observed between individual transcallosal neurons. The lack of any distance effect seen in the callosotomy with antibody infusion experimental sub-group argues against this possibility. The transcallosal neurons in this sub-group should in theory have experienced a complete and total blockage of contralateral signals due to the callosotomy, so that the only the ipsilateral axon target sites would have been affected by concentration dependent effects of the ipsilateral antibody infusion. The lack of difference in observed GAP-43 expression levels between neurons near the infusion site, where the concentration of antibody should have been highest, and those far away from the infusion site, where the concentration of antibody would have been lower, suggests that the range of diffusion of the antibody within the cortex in the individual animals was wide enough that concentration effects were not significant.

Our results are consistent with the findings of previous experiments involving DRG neurons where an up-regulation of GAP expression did not occur after central axotomy when the peripheral axon branch was left undamaged and allowed to act as a sustaining collateral. (Schreyer and Skene, 1993) Similarly, we found that GAP-43 expression was not up-regulated after any form of unilateral FGF-2 blockade, presumably because intact axons from the unaffected side continued to act as sustaining collaterals.

In DRG neurons, the peripheral axon appears to provide retrograde repression signals for both itself and the central axon. Peripheral axotomy alone results in an up-regulation of GAP expression and an improvement in the regenerative capacity of the central axon into permissive environments. (Richardson and Issa, 1984; Richardson and Verge, 1986) Our results with transcallosal neurons supports our hypothesis that in CNS neurons, multiple axon branches act as sustaining collaterals in concert, such that all must be disconnected before the retrograde repression of GAP expression is lifted, and that this difference in morphology accounts at least in part for some of the observed difference in regenerative capacity between PNS and CNS neurons.

Another difference observed between PNS and CNS neurons is the effect that the distance between the neuron cell body and the axotomy site has on regenerative capacity. In PNS neurons such as DRG cells (Liabotis and Schreyer, 1995) and spinal

motoneurons, (Fernandes et al., 1999) axotomy distance has no measurable effect on GAP expression or regeneration. In contrast, the few CNS neuron populations known to be capable of some regenerative response after axotomy invariably can only do so if the axotomy is proximal. For example, retinal ganglion cells in rats can regenerate axons into a permissive environment if the axotomy is within 3 mm of the eye but not beyond, (Doster et al., 1991) and rubrospinal neurons both up-regulate GAP-43 expression (briefly) and regenerate into a permissive environment following a cervical axotomy but not a thoracic one. (Fernandes et al., 1999)

The differing capacities for regenerative responses in CNS neurons after proximal versus distal axotomy has been attributed to the summation of negative signals provided by CNS myelin. The more distal the axotomy, the more CNS myelin and associated glial cells remain to provide more inhibition of regenerative responses. Our results suggest that neuronal morphology may provide an alternative or additional explanation. The more proximal the axotomy, the greater proportion of the neuron's axon collateral arborization is likely to be disconnected.

Another question that arises is whether or not the retrograde repressive acts as a summation of all signals received from the various axon collaterals, such that the greater the total signal, the greater the degree of inhibition of GAP expression, or instead in an all or nothing fashion with some specific threshold of signal above which GAP expression in suppressed. Experimental findings to date can be interpreted in both ways. In retinal ganglion cells, a proximal axotomy close to the eye would be highly likely to sever all of the axon collaterals, since the major collaterals branch off post-chiasmatically. (Felton and Jozefowicz, 2003) On the other hand, the rubrospinal tract innervates interneurons in the facial nerve nucleus and lateral reticular nucleus in the brainstem, and the anterior horn of the spinal cord in regions that project motor neurons to the muscles of the limbs. It is more difficult to determine whether or not a proximal axotomy at the cervical spine would interrupt the totality of retrograde signaling for any given rubrospinal neuron. It seems likely that a majority of axon collaterals would be disconnected, but the possibility would always remain that some axon collaterals still remain even more proximal to the axotomy.

Both explanations can also account for the variability in response observed in our experiments. Transcallosal neurons with widespread axon collaterals beyond the zone of antibody activity may have received varying amounts of intact retrograde signal. Alternately, different populations of transcallosal neurons may be responding differently to FGF-2, with different thresholds of sensitivity to FGF-2 blockade resulting in different levels of GAP-43 expression, and some TCNs may be insensitive to FGF-2, and are governed by a different retrograde repressor.

The average degree of elevation of GAP-43 expression we observed in our experiments was roughly twice the level seen in controls. This level is substantially lower than the levels of increase reported for some peripheral neurons, which are often ten or more times greater than unlesioned controls.(Bisby, 1988; Tetzlaff et al., 1991; Fernandes et al., 1999) This difference is consistent with the reduced ability of CNS neurons to mount regenerative responses after injury compared to PNS neurons. However, our GAP-43 expression levels were measured at a single time point, 7 days after experimental manipulation, so it is possible that GAP-43 expression was still climbing at that point and had not yet peaked, or had reached a higher level earlier and had started to fall. Also, the magnitude of the elevation of GAP-43 expression that was observed was the mean of a population with a wide variation in the levels of GAP-43 expression in individual cells. In the subpopulations with high levels of GAP-43 expression, the majority of TCNs had levels of expression that were three to five times control, which is consistent with the levels reported for rubrospinal neurons after proximal axotomy. (Fernandes et al., 1999) A few neurons were observed with levels of GAP-43 expression five to ten times controls, but no neurons were observed with levels of expression that exceeded ten times the control levels.

4.2 THE INHIBITION OF AXON REGENERATION IN THE CNS

4.2.1 Retrograde Repression as a Signal for Continuing Target Contact

Our results show that retrograde repression as a mechanism of regulation is active in the mature central nervous system, and previous experiments have demonstrated the same mechanism in the peripheral nervous system and the developing CNS.(Bisby, 1988;

Karimi-Abdolrezaee and Schreyer, 2002) So far, retrograde repression has been shown to regulate the gene expression of target neurons, reducing the expression of growth related genes, thus affecting the early phase of the initiation of regeneration after injury. In the peripheral nervous system, loss of the retrograde repressive signal indicates loss of target contact, and allows for the initiation of axon regeneration when target contact has been interrupted by injury. In the central nervous system, through the action of multiple sustaining collaterals, it acts to prevent the initiation of a regenerative phenotype after injury to some but not all of a neuron's axon collaterals.

Retrograde repression may play a role in the creation of appropriate functional axon connections during development and the maintenance of these same connections in the adult. It provides a stop signal to axon growth, such that developing axons do not overshoot their appropriate target tissues, and may provide some of the signals responsible for prompting a developing neuron to differentiate from a growth state to a functional state.

Our findings, along with previous work showing the successful induction of GAP expression by the interruption of retrograde axonal signaling alone (Liabotis and Schreyer, 1995; Bisby et al., 1996; Karimi-Abdolrezaee and Schreyer, 2002) suggest that retrograde repression plays a major role in the regulation of GAP expression in both the central and peripheral nervous systems. Other observations clearly demonstrate, however, that while GAP expression might be necessary, it is not, by itself, sufficient to ensure successful axon regeneration. (Richardson and Issa, 1984; Vidal-Sanz et al., 1987; Tetzlaff et al., 1991; Davies et al., 1997b; Moon et al., 2000) A permissive environment is also necessary to promote the outgrowth, extension, and guidance of regenerating axons after the GAP associated growth phenotype is reactivated. While the PNS generally provides such a permissive microenvironment, the mature CNS does not.

4.2.2 Inhibition of Regeneration Mediated by CNS Glia

Retrograde repression is not the only mechanism active in the adult CNS that inhibits axon outgrowth and regeneration after injury. CNS glia and its associated myelin plays a key role in the inhibition of axon outgrowth and regeneration. Axon outgrowth during development usually occurs in a myelin free environment, and CNS neurons

generally begin to lose the ability to robustly regenerate injured axons around the same time that myelination begins.(Qiu et al., 2000) Robust regeneration of mature CNS axons can be induced in tracts depleted of CNS myelin. (Moon et al., 2000) As previously mentioned, the increased effect of proximal axotomy on GAP up-regulation in CNS neurons can be alternately explained as a consequence of decreased total glial inhibition along the remaining short axon stump after a proximal axotomy compared to the longer axon stump with more associated glial cells and glial inhibition after more distal axotomy. Even in the PNS, mature myelin appears to have some inhibitory action. PNS axons only begin regenerating after myelin debris is cleared by Wallerian degeneration and the Schwann cells revert to a non-myelin producing phenotype.(Hall, 2005; Yiu and He, 2006) Unlike Schwann cells, oligodendrocytes and astrocytes in the CNS do not seem to revert to a growth-favoring phenotype after injury.

A number of factors associated with CNS glia that have inhibitory effects of axon outgrowth have been identified, including substances such as Nogo, myelin associated glycoprotein (which appears to promote neurite outgrowth during development but becomes inhibitory in the adult), semaphorin 4D, ephrin B3, and oligodendrocyte myelin glycoprotein.(Qiu et al., 2000; Yiu and He, 2006) Interestingly, many of these factors are sequestered within CNS myelin, and in the intact state, axonal exposure is limited. High levels of these inhibitors released into the CNS microenvironment by damaged myelin after injury.

In the injured state, the glial scar provides yet another level of inhibition of axon regeneration. Collateral axon sprouting from outside the area of injury has been observed, but even this limited attempt at regeneration invariably grows up to the site of injury but no further. Reactive astrocytes infiltrating the injury site may provide a physical barrier to axon regrowth, and also produce their own additional inhibitory factors. Experiments have shown that treatments that neutralize the activity of some of these inhibitors can reduce some of this inhibition and promote axon regeneration after spinal cord injury.(Moon et al., 2001) The result of all these inhibitory mechanisms is that the mature CNS microenvironment is normally significantly hostile to axon outgrowth and regeneration, allowing for only limited axon collateral sprouting and

plasticity, and after injury it becomes, almost paradoxically, even more profoundly inhibitory.

4.3 EVOLUTIONARY CONSIDERATIONS

Among vertebrates there is a clear pattern of progressive loss of regenerative capacity in the CNS over time. Anamniotic vertebrates such as fish, newts, and salamanders retain a robust ability to regenerate mature CNS axon tracts after injury. A reduction in regenerative capacity begins to be observed in anuran amphibians, such as frogs and toads, where long spinal cord axon tracts cannot regenerate after metamorphosis into the adult.(Skene, 1984) Amniotic vertebrates such as birds and mammals possess almost no ability to regenerate CNS axons beyond embryonic development, although some primitive mammals such as opossums which have abbreviated gestations and comparatively premature births are known to retain regenerative capability in the CNS for a few days after birth.(Yiu and He, 2006)

Why should higher vertebrates have lost the ability to regenerate CNS axons during the course of their evolution? The first question to consider is how much of an adaptive advantage the ability to regenerate CNS axons would actually be. Axonal regeneration, even in the most ideal circumstances, is a long-term mechanism. As vertebrates became larger and more complex, the consequences of any serious CNS trauma would have likely become ever more immediately catastrophic. For the average early mammal (or reptile or bird, for that matter), a severe CNS injury would have immediately resulted in an inability to obtain food or escape from predators, and would consequently have been fatal within a very short time. As such, any capacity to regenerate CNS axons over the long-term would not likely have provided a significant selective advantage. It is possible then, that the regenerative ability inherited by the first amniotes from their anamniote ancestors, became over the course of time a selectively neutral trait, which could then be freely lost by random mutation and genetic drift.

However, the presence of so many active and overlapping systems in the mature CNS that act in concert to suppress axonal regeneration strongly suggests that regenerative capacity was not merely passively lost over evolutionary time, but that growth restriction was actively favored by natural selection. That is, higher vertebrates

did not simply lose an ability to regenerate CNS axons that they no longer needed, but in fact gained the ability to restrict CNS axon regeneration as an adaptive evolutionary trait.

Why should the inability to regenerate a vital organ system be an adaptive response? Perhaps it arose as a secondary consequence of the development of another trait that proved beneficial. One possibility is that the inability for the adult CNS to regenerate axons is a consequence of the mechanisms controlling axon pathfinding during development. A common mechanism of axonal guidance during development is for attractive factors to draw developing axon terminals into an intermediate region between their origin and their ultimate target, after which the attractive factors change into repulsive ones that drive the axons out of the first intermediate region and towards the next, which produces its own attractive factors that in turn become repulsive. The process is subsequently repeated through any number of various intermediate areas until the final destination is reached. Thus, by the time of maturity, the majority of guidance signals along the axon tract are negative in nature, and many of these repulsive and inhibitory cues persist into adulthood.(Yiu and He, 2006) As a result, injured axons in the mature CNS are prevented from re-entering the same regions they traversed during development. Over evolutionary time, refinements in the specificity and precision of these guidance mechanisms would allow for the development of larger and more functional body plans and more sophisticated behaviors, and provide a strong adaptive advantage that would be favored by natural selection. The loss of regenerative capacities of limited survival value would, from an evolutionary perspective, be a small price to pay in exchange.

There may also be an intriguing link between axon regeneration and neuron survival after CNS injury. Mature cerebellar purkinje cells do not regenerate axons after injury, even if a permissive environment is provided, but are capable of long term survival after axotomy. In contrast, inferior olivary neurons whose axons are cut by the same lesion, up-regulate GAP expression and are capable of axon regeneration into a permissive environment after axotomy, but the vast majority die. (Dusart et al., 2005) In these neurons, the up-regulation of GAP-43 and c-Jun appear to be associated with a greater likelihood of cell death as well, suggesting that an attempt at axon regeneration and subsequent cell death are somehow linked. It is possible that the opposite may be

true, and the restriction of axon regeneration somehow promotes neuronal survival in injured CNS neurons. If this is the case, it may be that the survival of CNS neurons with complex axon arbors and multiple synaptic connections after trauma is favored by natural selection over the promotion of axon regeneration if that process also increases the risk of cell death and the subsequent loss of all its functional connections.

4.4 THE REGULATION OF PLASTICITY

4.4.1 The Role of Retrograde Repression in Regulating Neuronal Plasticity

Another trait that may have secondarily promoted an increasing restriction of regenerative capacity is the development of neuronal plasticity. All adaptive behavior is underpinned by plasticity within the CNS and constitutes a likely target for natural selection to act on. If axon regeneration is analogous to axon long tract elongation during development, then plasticity in the mature CNS is analogous to axon sprouting during target innervation and remodeling in development. The first process involves relatively rapid, straight and long process outgrowth, while the second is slower, and produces short processes with multiple branches.

Various GAPs are involved in both processes in both development (axon elongation and target innervation) and maturity (axon regeneration and reactive terminal arbor reorganization).(Wieloch and Nikolich, 2006) These two processes, however, are probably distinct, and regulated by different mechanisms.(Smith and Skene, 1997) Since some of the genes and proteins involved in the two processes are shared, it makes sense that their regulation be different and perhaps sometimes mutually antagonistic, such that the activation of one system does not inappropriately activate the other. Interestingly, in the previously mentioned cerebellar purkinje cells, short collateral sprouting is frequently observed after axotomy,(Dusart et al., 2005) suggesting a link between the promotion of plasticity and neuron survival.

In this light, the substances involved in retrograde repression are likely to have some role in plasticity regulation as well. Since they are thought to be produced by target tissues and act on the presynaptic cell, it would be highly surprising if they were not also

involved in plasticity in some way. At least some retrograde repressors in this view should simultaneously have opposing activities, promoting terminal arbor sprouting while inhibiting inappropriate long axon growth during periods of terminal arbor reorganization. Other retrograde repressors may also exist that act as inhibitors in both cases, helping to maintain stable functional connections over a long periods of time by limiting both continued long axon growth and unwanted collateral sprouting. It may even be possible that a single retrograde repressor may perform in both ways at differing times. In this scenario, retrograde repression of axon elongation would actually be a secondary function of a substance primarily involved in the regulation of neuronal plasticity.

The need for maintaining pre-existing functional connections and strictly regulating plasticity may be an important reason why regenerative responses are so limited in the central nervous system compared to the peripheral nervous system. Because CNS neurons elaborate many axon collaterals and participate in many interrelated neuronal circuits, it may be more important from the point of view of overall function to sustain pre-existing connections after traumatic injury than it is repair damaged axon connections, if the regenerative processes required to do so may as a side effect result in the disruption of other functional connections the neuron is participating in. After traumatic injury, sprouting has been observed to sometimes occur from intact axon collaterals distant from the injury site. (Grafstein, 1975) An instructive example are DRG neurons, where remote injury to the peripheral axon branch induces GAP up-regulation that can promote the extension of pre-existing and/or the growth of new central axon branches into permissive environments free of other inhibitory influences, even if there had been no initial injury to the central axons themselves. (Richardson and Verge, 1986) It is certainly possible that such new axon collaterals in a mature and functioning system could, if unregulated, form extraneous connections that might disrupt delicately balanced pre-existing circuits in a way that results in functional impairment. For example, the reactive sprouting of mossy fibers after a cortical insult has been implicated in the development of certain seizure disorders.(Chang and Lowenstein, 2003) Aberrant re-innervation after trauma can lead to poor functional outcomes even in the comparatively regeneration friendly PNS. Retrograde repression from sustaining

collaterals may be one mechanism that reduces the risk of this happening, since any neuron still possessing sustaining collateral axon connections is likely to be still participating in intact and useful circuits. From the point of view of the organism as a whole, it may be more beneficial to reduce the risk of developing significant functional distortion due to errant and/or overzealous axon regeneration in reaction to comparatively minor but common injuries than to promote robust regenerative capability after any devastating but rare injury that would significantly compromise immediate and short term survival irrespective of any potential for long term recovery.

The possibility that inhibitory processes play important regulatory roles has clinical implications for the treatment of CNS axonal injury. Certainly the risk exists that pharmacologically eliminating or counteracting these inhibitory mechanisms in an attempt to promote axon regeneration may have unexpected and undesirable secondary effects. An insufficiently specific and targeted attempt at treatment may well turn out to be akin to trying to restore a well-manicured garden that had been damaged in a fire by indiscriminately pouring fertilizer onto it. There is much potential for unintended consequences, and inadvertently promoting the development of a seizure disorder is only one possibility. Many higher level functions may turn out to be dependent on the strict regulation of the plasticity of the underlying neuronal circuitry, and highly sensitive to even small disruptions. However, the evolutionary consideration also raises the intriguing possibility that evolutionarily primitive but desirable functional capabilities such as improved bowel and bladder control, or independent respiration after severe high spinal cord injury(Yiu and He, 2006) may be comparatively robust and would be preferentially preserved by relatively simple manipulations of the inhibitory mechanisms preventing CNS axon regeneration.

4.4.2 The Role of GAP-43 and FGF-2 in Neuronal Plasticity

As previously mentioned, the developmental downregulation of GAP-43 expression is biphasic, with decreasing expression occurring when growing axons first contact target tissue, and later upon the maturation of the terminal arborization. (Karimi-Abdolrezaee et al., 2002) This suggests that GAP-43 is involved in both the process of axon elongation and the later process of terminal branching, and supports the hypothesis

that the two processes are regulated by distinct mechanisms. Given this role in terminal arborization, it is not surprising that GAP-43 has been implicated in neuronal plasticity in the mature CNS.(Strittmatter et al., 1992; Benowitz and Routtenberg, 1997)

Interestingly, there is evidence to suggest that FGF-2 is also involved in neuronal plasticity in the adult, and that it acts to promote the process. Studies have shown that treatment with FGF-2 can promote the recovery of sensorimotor function after MCA infarction in rodents, when administered both immediately after the ischemic insult, or later in a delayed fashion.(Kawamata et al., 1996) Early administration of FGF-2 appears to enhance neuronal survival and the proliferation of neuronal stem cells, and results in a reduction of the size of the infarct. However, functional improvement can also be seen after late administration, in which case the infarct size does not change. In this case, an increase in GAP-43 is observed in the ipsilateral peri-lesional area, and in the contralateral sensorimotor cortex, suggesting the possibility that the functional improvement is due to the plastic development of compensating collateral connections.(Kawamata et al., 1997) Furthermore, this functional improvement does not occur if GAP-43 function is silenced with anti-sense mRNA.(Kawamata et al., 1999)

Comparing these results with our results leads to some interesting speculation. In both cases, an increase in GAP-43 expression was observed, but in one case this was as a result of FGF-2 administration, while in the other, it was a response to FGF-2 blockade. One possibility is that these seemingly paradoxical results are due to differences in the experimental situations. In the experiments of the Kawamata group, the increase of GAP-43 expression was seen in brains subject to widespread ischemic injury, while our experiments involved intact cortex. Metabolic changes as a result of ischemia may have altered the normal neuronal response to FGF-2, or FGF-2 may have promoted the survival of neuronal populations with higher baseline GAP-43 expression, resulting in a relative increase in GAP-43 levels. In the Kawamata experiments, FGF-2 was administered intracisternally, and presumably distributed throughout the CNS, while in our experiments, FGF-2 activity was blocked in a local cortical area. As in the case of the positive response to FGF-2 administration in frog retinal ganglion cells, dosage related effects, wherein one case a relatively high level of FGF-2 is exogenously administered, while in the other case the activity of a low level of endogenous FGF-2 is

blocked, may have contributed to the difference in results. The Kawamata group also quantified GAP-43 expression using GAP-43 protein rather than GAP-43 mRNA. Since GAP-43 is transported to and concentrated in elongating axon terminals, it is not clear whether or not the observed GAP-43 protein was produced in the local cortex or transported there from distant sites induced by the global exposure to FGF-2. Either way, the specific neuronal population in which GAP-43 expression is up-regulated is not known, so the elevated GAP-43 levels observed may not have been produced by transcallosal neurons, but by another neuronal population that responds to FGF-2 in an opposite fashion.

Another possibility worth considering is that different results were observed because different mechanisms were investigated. That is, in the Kawamata experiments, GAP-43 was up-regulated by FGF-2 in relation to the promotion of neuronal plasticity, while in our experiments, FGF-2 was acting to inhibit GAP-43 expression in relation to limiting axon elongation. If this is true, then FGF-2 may be one of the postulated substances with a dual role, acting to enhance collateral sprouting in relation to plasticity, while also inhibiting long tract regeneration as a retrograde inhibitor. In this light, the activity of FGF-2 in developmental axon guidance is suggestive. Its ability to inhibit the forward movement of the primary growth cone in order to promote the development of collateral branching(Szebenyi et al., 2001) is exactly the kind of mechanism one might expect to see in a substance that acts both as a retrograde repressor of axon long tract regeneration and a promoter of collateral sprouting in plasticity.

Another possible resolution of the apparent conflict in our results is seen when the areas of cortex where GAP-43 up-regulation was observed is compared between the Kawamata experiments and our experiments. The area of the MCA territory infarction zones in the Kawamata experiments included within it the area of contralateral minipump implantation in our experiments. Up-regulation of GAP-43 was seen ipsilaterally in the motor cortex medial to the infarction zone, and contralaterally in the homologous medial motor cortical area. However, elevated GAP-43 levels were not detected contralateral parietal cortex that was homologous to the infarct site,(Kawamata et al., 1997) which was the area that we studied, even though the infarct in the other hemisphere destroyed the transcallosal target site. This is exactly what one should expect if our hypothesis that

FGF-2 represses GAP-43 expression in transcallosal neurons in the parietal sensory cortex is correct. The Kawamata results also suggest a possible explanation for the trend we observed towards slightly higher GAP-43 expression in medial TCNs compared to lateral TCNs. The mean levels of GAP-43 expression may have been increased in some of the medial neurons due to a plasticity reaction to the infusion site trauma.

Taken together, the results of our experiment and those reported by the Kawamata group may be demonstrating that FGF-2 has opposite effects on two neighbouring regions of sensorimotor cortex, promoting GAP-43 expression in the medial and largely motor areas while repressing GAP-43 in the more lateral and largely sensory areas. FGF-2 may therefore by involved in keeping some of the axon tracts related to motor and sensory cortex separate, preventing inappropriate cross connections between the motor and sensory areas of the brain.

4.5 FUTURE DIRECTIONS

4.5.1 The Rat Transcallosal Neuron as an Experimental Model and Proposed Refinements of the Experimental Technique

These experiments demonstrate the usefulness of the TCN of the rat as an experimental model for studying the regulation of GAP expression in the mammalian CNS. The TCNs were easily identified using retrograde fluorescent tracers. Although the fluorescent stain had a tendency to be washed out of the sections during subsequent processing, necessitating the creation of fluorescent cortical maps prior to in situ hybridization, this proved to be only a minor technical inconvenience.

The homologous nature of the transcallosal projection (Cracco et al., 1989; Swanson, 2003) allowed for the specific targeting of both ipsilateral and contralateral axon termination sites through the use of stereotactically implanted osmotic mini-pumps. One problem encountered during mini-pump implantation in these experiments was the imprecision involved in separately implanting the ipsilateral and contralateral mini-pump brain cannulae in the bilateral infusion experiments. Motion of the brain cannulae after placement but prior to complete fixation during manual implantation also contributed to increased amounts of tissue damage and scarring at the infusion site.

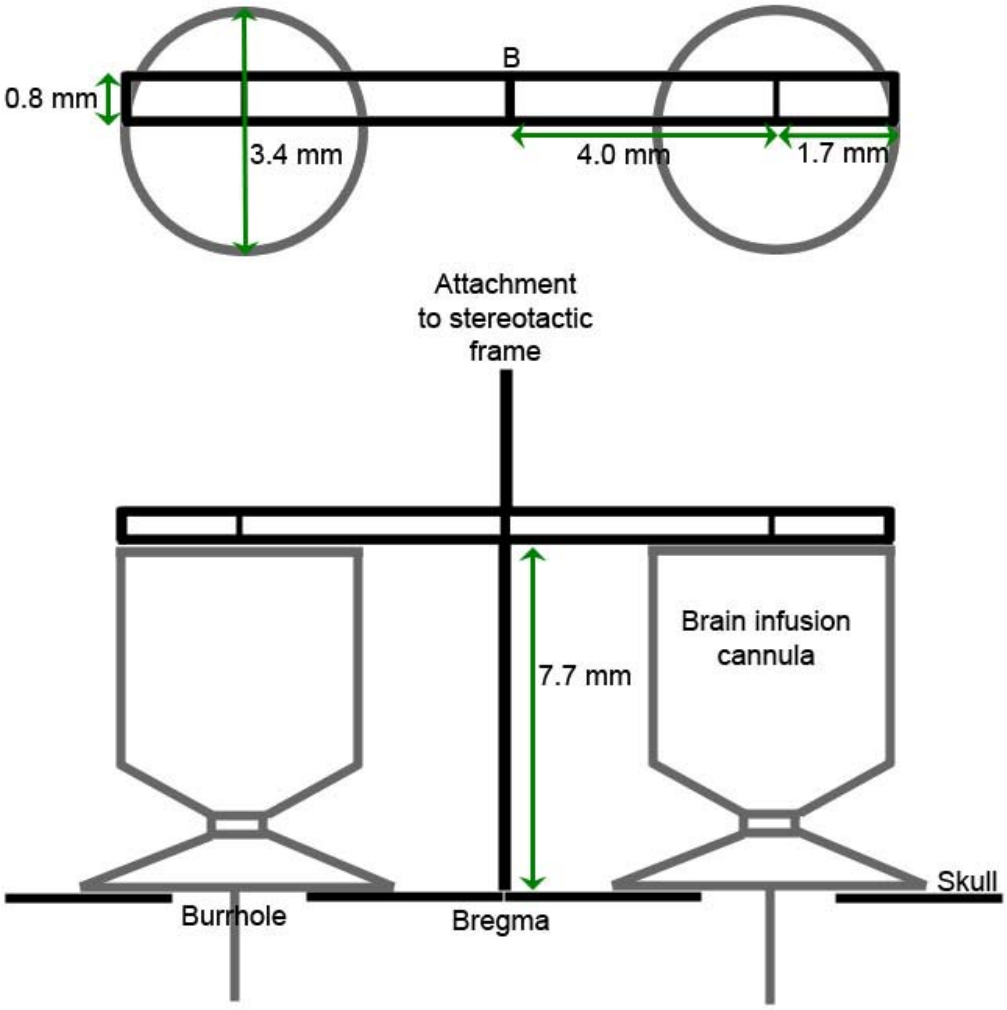

Figure 4.1: Proposed scaffold for bilateral mini-pump implantation. View from above (top) and view from side (bottom). Brain infusion cannulae are attached to scaffold, and entire construct stereotactically implanted in a single step, with point B positioned over Bregma. Scaffold then detached along with detachable knobs of Alzet osmotic mini-pump brain infusion cannula head.

For future bilateral infusions, both brain infusion cannulae can be attached prior to implantation to the ends of a sterile scaffold, as shown in figure 4.1. The scaffold can be made of wood or steel wire, or possibly even hard rubber, which would allow it to be pierced by a syringe needle which could subsequently be attached to the stereotactic frame arm. After attachment to the stereotactic arm, the entire construct is positioned with its center over the midline at the desired AP distance relative to Bregma, allowing

both the ipsilateral and contralateral brain infusion cannulae to be placed simultaneously, and held motionless by the frame until fixed into place by the polyacrylamide glue, after which the scaffold can be detached from the implanted brain cannulae. The shape and size of the scaffold can be modified to allow for implantations if different sites are required or animals of different sizes are used.

The corpus callosum proved to be a readily accessible site for experimental lesioning. In these experiments, partial callosotomies were performed, with only the anterior corpus callosum sectioned, and the posterior portion left intact in order to minimize injury to midbrain structures lying directly below the posterior third of the body of the corpus callosum. Extending the callosotomy to include the posterior portion of the corpus callosotomy would be technically relatively simple, although it may carry an increased risk of morbidity to the experimental animals.

The assumption was made that the majority of transcallosal fibers projecting from the cortical area of interest would cross the midline without first traveling parallel to the corpus callosum any significant distance in the anterior-posterior plane, such that axons originating from neurons in the parietal cortex around -0.8 mm to Bregma would cross in the corpus callosum within the anterior-posterior extent of the callosotomy section (from 1.2 mm to Bregma to -2.8 mm to Bregma, or 4 mm total centered on -0.8 mm to Bregma), such that the anterior callosotomy would be sufficient to section the majority of these axons. If there was any significant subpopulation of axons that did not do this, but traveled posteriorly for a significant distance and crossed the midline in the posterior third of the corpus callosum, then these axons may account for some of the neurons that were observed to remain with low levels of GAP-43 expression in the callosotomy experiments, since these neurons would not have had their contralateral FGF-2 signaling interrupted by the callosotomy. However, FGF-2 signaling in these neurons should still have been interrupted by contralateral antibody infusion, so these neurons would still have been exposed to bilateral FGF-2 blockade in the bilateral antibody infusion experiments. The fact that no significant differences in the levels of GAP-43 expression were observed between the bilateral infusion and callosotomy with infusion experimental sub-groups argues against neurons with this sort of axon morphology having a significant impact on the overall results.

The degree of tissue damage in the midline cortex associated with callosotomy in these experiments was not insignificant. However, the presence or absence of a callosotomy scar did not produce any noticeable effects in these experiments. The level of GAP-43 expression in the experimental subgroup receiving callosotomy without antibody infusion was not significantly different than the control groups that did not have callosotomies. The two animals that had incomplete callosotomies also did not have GAP-43 expression levels substantially different from the controls, even though the size of the callosotomy scar in these animals was essentially no different from animals receiving complete callosotomies, except for the absence of complete transection of the corpus callosum itself. Finally, there was no significant difference between the experimental subgroup that received callosotomy and antibody infusion and the subgroup that was treated with bilateral antibody infusion, in which there was no callosotomy scar.

The size of the midline callosotomy scar could be reduced in future experiments by using a finer callosotomy knife. Alternatives to the 25 gauge syringe needle used in these experiments that can be considered include a stereotactic wire knife, a fine scalpel blade such as a Beaver blade, or simply a finer gauge syringe needle.

Although not attempted in this study, methods are also available for isolating and culturing transcallosal neurons, allowing them to be studied under controlled in vitro conditions.(Catapano et al., 2004) Other potential substances of interest besides FGF-2 can have their effects on transcallosal neurons studied in vitro prior to in vivo experimentation.

Aside from being just an experimental model, the transcallosal neurons themselves are a subject on which further scientific study is warranted. In addition to being prototypical excitatory pyramidal projection neurons, they are subject to dysgenesis and degeneration in a variety of conditions, with many implications for human health.(Catapano et al., 2004) From a clinical perspective, injury to the corpus callosum in humans is often seen in the setting of head trauma, and may also occur as a consequence of the rupture of midline aneurysms, or infarction in the territories of the distal branches of the anterior cerebral arteries. The deliberate transection of the corpus callosum is a neurosurgical procedure sometimes indicated for the improved control of intractable seizures, and disconnection syndromes are a recognized adverse outcome,

particularly if the posterior third of the corpus callosum is damaged. Neurosurgeons also use transcallosal approaches to access pathology involving the lateral and sometimes the third ventricles, and inadvertent injury to portions of the corpus callosum can occur in these procedures.

4.5.2 The Effect of Time on Neuronal Response to Bilateral FGF-2 Blockade

In these experiments, an increase in GAP-43 expression in response to bilateral FGF-2 blockade was observed at a single time point, 7 days after the initiation of the blockade. The timing of GAP responses after axonal injury has been documented in other known experimental systems. In frog and goldfish retinal ganglion cells, GAP-43 synthesis begins to increase 4 to 5 days after injury and reach peak levels 10 to 14 days after injury, and fall back to background levels 5 to 10 weeks after axotomy, usually after axon regeneration is complete.(Skene and Willard, 1981b; Skene, 1984) Levels of GAP-43 in rat retinal ganglion cells were observed to be elevated between days 6 to 26 after intraorbital axotomy near the neuron cell body.(Doster et al., 1991) In rat facial motorneurons, GAP-43 mRNA expression peaked at 7 days post lesioning and remained at that level for more than three weeks, while in rubrospinal neurons, GAP-43 expression also peaked at 7 days after injury, and remained elevated even at 7 weeks after injury, but declined from the peak level of expression as the neurons were observed to atrophy.(Tetzlaff et al., 1991)

This suggests but does not confirm that the level of GAP-43 expression measured 7 days post FGF-2 blockade in our experiments would be likely at or near the peak of GAP-43 up-regulation. In order to investigate the timing of GAP-43 up-regulation after FGF-2 blockade, bilateral antibody infusion and callosotomy with antibody infusion experiments can be performed with infusion times of varying length. Based on the available data from other neuron populations, potential time intervals worth investigating might be 3 days, 7 days, 2 weeks, 4 weeks, and 6 weeks, although longer time intervals may require several interval replacements or refilling of the implanted mini-pumps. In this fashion, it can be determined how soon after FGF-2 blockade is the up-regulation of GAP-43 is initiated, how quickly GAP-43 mRNA levels rise, when and at what level does it peak, how long GAP-43 expression is sustained at high levels, and how long, if

ever, it takes for levels of GAP-43 expression to decrease back to baseline levels. Comparisons can be made between bilateral antibody infusion and callosotomy combined with ipsilateral antibody infusion to determine if there are any differences in the timing and magnitude of the response between the two groups. Further information can also be obtained with regards to neuronal survival and atrophy over time.

4.5.3 Other GAPs and Other Proteins

In these experiments, GAP-43 was used as a marker for GAP expression in general, on the assumption that increased levels of GAP-43 expression would be accompanied by increased levels of expression of other growth associated proteins. Because GAP-43 and FGF-2 both appear to be involved in both axon regeneration and plastic collateral sprouting, GAP-43 may not necessarily be the ideal marker to sort out the activity of these two processes in the TCN system. Thus the effect of bilateral FGF-2 blockade on the levels of expression of mRNAs of other GAPs would be worthwhile. This can be easily done by using cDNA probes for other GAPs for in-situ hybridization. Candidates for investigation would include GAP-24 (Skene and Willard, 1981a), alpha and beta-tubulin (Skene and Willard, 1981a).

The most interesting to investigate may be the GAPs that act as transcription factors, such as c-Jun (Jenkins and Hunt, 1991), Jun-D, and activating transcription factor 3, since these proteins are probably involved in the coordination of the generalized growth response.

The expression of non-GAP proteins known to be produced by mature functioning TCNs would also be worth investigating to see what other effects on the normal TCN phenotype bilateral FGF-2 blockade might have. Such substances might include peptide neurotransmitters known to be produced by TCNs such as Substance P, (Conti and Manzoni, 1994) or the metabotropic glutamate receptor. Since transcallosal neurons continue to maintain axon collaterals to local targets after distal axotomy, and the possibility that retrograde repression may play a role in the regulation of plasticity and the maintenance of functional connections, it would be interesting to investigate whether bilateral FGF-2 blockade would result in the down-regulation of proteins associated with the mature and functional neuronal phenotype.

4.5.4 Subpopulation Investigation

It was clear that individual transcallosal neurons were responding differently to the FGF-2 blockade in our experiments, and that the varying responses were falling in a gradient from low to high levels of GAP-43 expression. The population histograms did not reveal any obvious second peaks that might have identified any one specific sub-population of neurons that were particularly responsive, or non-responsive, to FGF-2 blockade. The possibility remained that several sub-populations of transcallosal neurons exist with differing degrees of sensitivity to retrograde repression from FGF-2.

Attempts can be made to identify specific subpopulations within the greater parietal TCN population, which would allow for comparison of GAP-43 mRNA levels between these subpopulations, if they exist. Like most pyramidal neurons, the neurotransmitters of TCNs are primarily excitatory, and are glutaminergic. Some transcallosal neurons are glutamate negative, and these may use aspartate as a neurotransmitter instead.(Conti and Manzoni, 1994) Specific stains or antibodies for glutamate, aspartate, peptide neurotransmitters such as the previously mentioned Substance P and cholecystokinin, or their metabolic precursors, could be used to identify transcallosal neuron subpopulations using these neurotransmitters. Antibodies to different FGF-2 receptors could be used to see if there are differences in the expression of these receptors among different subpopulations of TCNs.

4.5.5 Axon Outgrowth after FGF-2 Blockade

As previous studies have shown, in the central nervous system, even if GAP expression is successfully up-regulated following axonal injury, significant axon outgrowth generally does not occur in the absence of a permissive environment.(Vidal-Sanz et al., 1987; Doster et al., 1991; Fernandes et al., 1999) It would therefore be interesting to see if ipsilateral FGF-2 blockade after callosotomy can in addition to up-regulating GAP expression, also induce axon outgrowth into and past the callosotomy scar site. A variety of methods are available for assessing and quantifying neurite outgrowth and axon regeneration. Histological examination with anterograde tracers such as biotinylated dextrane amine can be used to identify axon sprouting and the

generation of new growth cones and the extent of axonal regeneration across the corpus callosum scar can be followed over time. The detection of specific proteins transported to and concentrated in growth cones beyond the scar site along the course of the transcallosal fiber tract could also be used to assess the extent of axon regeneration over time. Candidate proteins would include GAP-43 itself and alpha-1-tubulin.(Skene and Willard, 1981b; Miller et al., 1989)

Given the known inhibitory effects of CNS glia and glial scar tissue, it is likely that GAP-43 induction alone would be insufficient to cause axon regeneration across a callosotomy scar to any significant extent. If this is indeed the case, attempts could be made to promote axon growth across the callosotomy site by combining FGF-2 blockade with modulation of known glial inhibitors, in order to create a permissive environment for axon regeneration. For example, the neutralization of the glial inhibitor nogo by the monoclonal antibody IN-1 has been shown to facilitate generative responses in lesioned CNS fiber tracts.(Bandtlow and Schwab, 2000; Qiu et al., 2000) IN-1 could be infused directly into the callosotomy scar site using a second osmotic mini-pump in conjunction with ipsilateral FGF-2 blockade. Alternately, the nogo receptor itself or its downstream signaling pathways might be inhibited. Another substance that could be tested would be chondroitinase ABC (Moon et al., 2001) which has been shown to counter the inhibitory effects on axon regeneration of chondroitin sulfate proteoglycans produced by reactive astrocytes. Other alternatives that could be tried would include implanting Schwann cells into the callosotomy scar, or bridging the callosotomy scar with a peripheral nerve graft.

4.5.6 Transcallosal Neurons in Other Areas of Neocortex

The effect of bilateral FGF-2 blockade can be investigated in TCNs in areas of cortex other than the anterior parietal area chosen for these experiments. Other cortical areas of interest can readily be investigated by changing the coordinates of the mini-pump implantations. More posterior parietal cortex, for example, can be targeted using coordinates 5.0 mm lateral to midline and 2.0 mm posterior to Bregma.

Motor cortex in the frontal lobe would be one area of interest for further investigation. In these experiments there was a trend towards medial neurons having higher levels of GAP-43 expression after bilateral FGF-2 blockade that did not reach

significance. Because motor cortex is found just medial to the parietal infusion site area used in these experiments, one explanation for this finding would be that motor cortex TCNs respond to bilateral FGF-2 blockade with a higher level GAP-43 mRNA up-regulation, and the observed increase in mean GAP-43 mRNA expression seen in medial neurons is due to the presence of some motor cortex TCNs in this subpopulation. Frontal motor cortex in the rat can be targeted using coordinates 3.0 mm lateral to midline and 1.0 mm anterior to Bregma. Motor cortex can also be differentiated histologically from parietal cortex because cortical layer IV is absent in motor cortex but prominent in parietal cortex.(Swanson, 2003)

Another cortical area of interest would be the cingulate cortex, which lies just medial to midline. Because of the transcallosal neurons in cingulate cortex are a developmentally distinct subpopulation that extends midline crossing axons earlier and act as pioneering fibers for the remainder of the transcallosal projection, it would not be surprising if they had a different response to signaling molecules such as FGF-2. With evidence suggesting that these neurons elaborate axons that act as pioneering fibers for the remainder of the transcallosal projection (Richards, 2002), it might be hypothesized that cingulate transcallosal neurons would possess more robust regenerative capacities, and perhaps retain these greater capacities into maturity. Targeting the cingulate cortex with osmotic mini-pumps would be more difficult however, because the minimum lateral distance to midline at which bilateral mini-pumps could be implanted would be 1.7 mm due to the diameter of the brain infusion cannula (Alzet™). Also, the callosotomy technique used in these experiments produces significant damage and scarring in the ipsilateral cingulate cortex, and as a result would not be suitable for experiments investigating cingulate neurons of any kind.

4.5.7 The Mechanism of Action of FGF-2

The experimental results presented here leave open the question as to the precise mechanism by which FGF-2 produces the effect of retrograde repression. In the adult CNS, FGF-2 has been found in both neurons and glia, widely distributed throughout the brain.(Reuss and von Bohlen und Halbach, 2003) We confirmed the presence of FGF-2 in parietal cortex using Western blot. The specific neuron and glial cell populations

producing FGF-2 in the cortex can be identified with in-cell Western blot and in situ hybridization of FGF-2 mRNA.

Four FGF receptors have been characterized to date.(Yazaki et al., 1994; Chadashvili and Peterson, 2006; Maric et al., 2007) All four receptors are tyrosine kinases, and FGF-2 demonstrates high affinity for all of them.(Reuss and von Bohlen und Halbach, 2003) In the adult CNS, FGFR1 has been found in neurons(Leadbeater et al., 2006) while FGFR2 has been found in astrocytes.(Chadashvili and Peterson, 2006) FGFR3 has been reported to be expressed in glial cells diffusely throughout the brain, and FGFR4 does not appear to be expressed in the mature CNS.(Yazaki et al., 1994; Reuss and von Bohlen und Halbach, 2003) FGFR1 has also been associated with long-term potentiation, memory consolidation and neurogenesis.(Zhao et al., 2007) If the promotion of plasticity and retrograde repression are separate and distinct functions of FGF-2, then one might hypothesize that the retrograde repressive function of FGF-2 may be mediated by FGFR2 and/or FGFR3, though this is far from certain, because one can also hypothesize that the retrograde repressor should be produced directly by the target neuron itself, which would implicate FGFR1. Of course it is also possible that FGF-2 acts indirectly through a variety of paracrine and/or autocrine effects to stimulate the production of a second substance that acts as the direct retrograde repressor, in which case the action of FGF-2 in this regard could be mediated by any of the three receptors alone or in combination.

To determine which of these receptors are responsible for the retrograde repression effect of FGF-2, bilateral infusions of function blocking antibodies, or other inhibitor, of these receptors can be infused using the same experimental methods described here, either individually, or in combination, to determine if the elevation of GAP-43 expression seen with FGF-2 blockade can replicated by FGFR blockade. Another possible target of investigation would be heparin sulfate proteoglycans, which act as co-receptors.(Leadbeater et al., 2006)

Second messenger systems implicated in FGF-2 signaling include PLCγ, src, Crk, SNT-1/FRS2.(Reuss and von Bohlen und Halbach, 2003) Also, cyclic AMP has been shown to play a role in GAP-43 expression and axon outgrowth in both the CNS and

PNS.(Andersen et al., 2000a; Andersen et al., 2000b) All of these pathways constitute promising candidates for future investigations.

4.5.8 The Effect of Bilateral FGF-2 Blockade on Non-transcallosal Neurons

One interesting incidental finding of our experiments was the observation of neurons that displayed high silver grain density, suggesting high GAP-43 mRNA expression, that were not labeled with the retrograde fluorescent dye as TCNs. The identities of these neurons remain an open question. Some of them are likely to be TCNs that, for whatever reason, were not labeled successfully by the fluorescent dye. Others may belong to other neuron subpopulations that are sensitive to FGF-2. It would be expected, if our hypothesis is correct, that interneurons with all their axon targets in the local cortical area that utilize FGF-2 as a retrograde repressor should display elevated levels of GAP-43 mRNA expression even with an ipsilateral FGF-2 blockade, and such neurons may account for some of the minority of neurons that were found to have apparently elevated GAP-43 mRNA expression in all experimental sub-groups with ipsilateral or bilateral FGF-2 blockade. Also, a small number of neurons may have had, by chance, all their axon collaterals on both sides of midline injured as a result of the tissue damage caused by the bilateral mini-pump cannulae, and these neurons may have responded to the injury by up-regulating GAP-43 mRNA expression. Finally, there may exist a small population of neurons in the parietal cortex with constitutively elevated levels of GAP-43 mRNA expression regardless of the effects of FGF-2.

Nevertheless, our qualitative observations seem to indicate that the number of non-transcallosal neurons that up-regulate GAP-43 mRNA expression and the magnitude of the up-regulation are both substantially increased in response to bilateral FGF-2 blockade. This raises the possibility that our observed response to bilateral FGF-2 blockade is not specific to transcallosal neurons, but instead may be a general response of many different types of cortical neurons. However, the fact that the response of non-transcallosal neurons, like that of the TCNs, was much stronger with bilateral FGF-2 blockade than unilateral FGF-2 blockade means that the responding non-transcallosal neurons must have a mechanism for the communication of signals and the coordination of responses across the midline. The strongest candidates available for providing this

mechanism are the TCNs themselves. A reasonable hypothesis worth testing would be that, as part of their overall response to bilateral FGF-2 blockade, TCNs mediate secondary signals that result in the up-regulation of GAP expression in other neighbouring cortical neurons. These signals may potentially be transmitted via anterograde local axon connections, by the secretion of diffusible paracrine factors, or through glial intermediaries, among other possibilities.

4.6 CONCLUDING THOUGHTS

In these experiments, retrograde repression has been demonstrated, for the first time that the author is aware of, to function in the adult mammalian CNS, supplementing previous evidence for this mechanism in the PNS(Bisby, 1988; Schreyer and Skene, 1993) and embryonic central nervous system. (Karimi-Abdolrezaee and Schreyer, 2002). Unlike the many previously elucidated glial based mechanisms of regeneration inhibition, retrograde repression is primarily a neuronal mechanism. The effects of retrograde repression are dependent on neuronal properties and morphology. The localization of the retrograde repressive effects to the specific target tissues of the neuron in question strongly suggests that the neuronal synaptic targets themselves are involved in mediating the repressive signal. It is not yet clear whether or not the repressive signal is sent directly from the post-synaptic to the pre-synaptic neuron, or indirectly through associated glial cells perhaps responding to primary signals from the post-synaptic neuron.

A significant change in gene expression was observed in these experiments after the cortical infusion of a single pharmacological agent. If our findings are supported by further investigation, this has significant implications for the possibilities of future clinical intervention. The implantation of devices capable of delivering controlled doses of pharmacological agents into precise locations even deep within the brain is readily achievable with modern neurosurgical technique. On the other hand, our results also imply that it might be necessary to manipulate the microenvironments of multiple areas of the CNS distant to the injury site in order to promote the regeneration of CNS axons after trauma. For example, in order to overcome the effect of sustaining collaterals in the

corticospinal tract if one were trying to promote axon regeneration after spinal cord injury, it might be necessary to target all the axon collaterals of the involved neurons in the motor cortex, which terminate in disparate sites throughout the central nervous system which may include adjacent cortical areas, the basal ganglia, and the brainstem. Nevertheless, this kind of stereotactic targeting of multiple sites, while technically challenging, is not, in theory, infeasible.

Another intriguing speculation is the question of whether or not the effects of pharmacologic manipulation may be replicated in whole or in part by electrical stimulation. This would open up a whole realm of possibilities with regards to functional neurosurgery and the implantation of electrodes into axon target sites with the attendant advantages of programmability and reversibility.

Of course, manipulating retrograde repression alone is unlikely by itself to result in clinically significant functional outcomes, and promoting desirable gene expression is only the first step of the many required to achieve successful axon regeneration. The glial mechanisms of growth inhibition must also be addressed. Any successful future treatment for central nervous system axonal injury will likely be multidirectional and aimed at a wide range of targets, both physical and biochemical.

Finally, in addition to the role it plays in limiting axonal regeneration in the adult CNS, retrograde repression likely plays important roles in the establishment of neuronal circuitry during development and the modulation and regulation and neuronal plasticity in maturity. Further study of this mechanism holds the promise of shedding new light on all of these subjects.

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APPENDIX A

SOLUTION RECIPES

General Stock Solutions

4% Paraformaldehyde

For 1 L: 40 g paraformaldehyde
500 mL distilled water
500 mL 2X PBS

1. Dissolve paraformaldehyde in water while heating to 70°C.
2. Add 10N NaOH dropwise until solution is clear
3. Add 2X PBS
4. Filter to remove suspended particles

Phosphate Buffer Stock (PB)

30.8 g NaOH
134.6g $NaH_2PO_4 \cdot H_2O$
Double distilled water up to 2 L

1. Dissolve NaOH first
2. Adjust final pH to 7.4

Phosphate-Buffered Saline (PBS) 2X

17.0 g NaCl
1 L PB
Double distilled water up to 2 L

1 M Tris-HCl pH 7.6

121.1 g Tris base
800 mL water

60 mL HCl

500 mM EDTA

1.86g EDTA

10 mL water

0.2 g NaOH, adjust to desired pH

Proteinase K stock 20 mg/mL

5 mL sterile water

100 mg proteinase K

Store at -20°C in 200 μL aliquots

Proteinase K 20 μg/mL

For 200 mL: 10 mL 1M Tris-HCl pH 7.6

2 mL 500 mM EDTA

200 μL proteinase K stock 20 mg/mL

188 μL double distilled water

In Situ Hybridization Solutions

Hybridization Cocktail

50 mL deionized formamide
20 mL 20X SSC
1 mL 100X Denhardt's Solution
10 mL 0.2M $NaPO_4$ pH 7.0
10 g dextran sulfate
5 mL 20% sarcosyl (Sigma L5125)

1. Dissolve while stirring
2. Add sarcosyl last to avoid foaming
3. Filter through 0.45 μm filter
4. Store at -20°C in 5 mL aliquots

SSC (20X)

175.3 g NaCl dissolved
88.2 g Na citrate
1L double distilled water

1. Dissolve NaCl in 800 mL double distilled water
2. Add Na citrate
3. Adjust pH to 7.0 with 10N NaOH or HCl
4. Add double distilled water to 1 L
5. Autoclave

Deionized Formamide

Formamide (Fischer Scientific)
Mixed bed ion exchange resin (Biorad AG 501-X8

1. Gently stir required volume of formamide with ion exchange resin
   (1 g exchange resin per 10 mL formamide)
2. Store at -20°C in 5 - 15 mL aliquots

Denhardt's Solution (100X)

5 g Ficoll (Sigma, molecular biology grade)
5 g polyvinylpyrollidone (Sigma, molecular biology grade)
5 g Bovine Serum Albumin (Pentax Fraction V)
Double distilled water to 250 mL

1. Filter through 0.45 μm Nalgene filter
2. Store at -20°C in 2.5 mL aliquots

5 M Dithiothreitol (DTT)

15.45 g DTT (Sigma, molecular biology grade)
20 mL 0.01 M Na acetate, pH 5.2

1. Dissolve DTT in Na acetate
2. Filter through 0.45 μm Nalgene filter
3. Store at -20°C in 1 mL aliquots
4. Do not autoclave any solutions containing DTT

Salmon Sperm DNA (Sigma D 1626)

1. Dissolve salmon sperm DNA in 100 mL water treated with DEPC
2. Seal with parafilm and stir overnight at room temperature
3. Aliquot into 1 mL volumes in sterile Eppendorf tubes
4. Sonicate to disrupt DNA
5. Store at -20°C

Western Blot Solutions

Whole Cell Extract Lysis Buffer

Final Concentration	For 100 mL
20 mM HEPES pH 7.5	2 mL 1 M stock
50 mM KCl	1.7 mL 3 M stock
10% glycerol	10 mL 100% stock
0.5 mM EDTA (RNAse free)	100 μL 0.5 M stock
0.5 mM EGTA (RNAse free)	100 μL 0.5 M stock
1 mM DTT	100 μL 1 M stock
1X Anti-protease cocktail (Sigma)	1 mL 100X stock

1. Add DTT prior to use. DTT remains active up to 3 months frozen
2. Store at -20°C to -80°C

Polyacrylamide Gel Tank Buffer

12 g Tris Base
57.6 g glycine
4 g SDS
Deionized water to 4 L

Sample Loading Buffer (1X)

1 mL 10% SDS
0.1 mL 1 M Tris pH 7.5
2.0 mL glycerol
6.9 mL deionized water
Add a few crystals of bromophenol blue

Gel Solution A

61.2 g Tris Base
11.05 g Tris-HCl
0.92 mL TEMED
Add deionized water to 400 mL

pH to 8.9 with HCl

Gel Solution B

12.1 g Tris Base
1.6 mL TEMED
Add deionized water to 200 mL

pH to 6.9 with HCl

Running Gel (12%)

For 1-4 gels:
15 mL Gel Solution A

18 mL 40% acrylamide/1.2% Bis
0.6 mL SDS
26.4 mL deionized water

Degas under vacuum (do not allow to foam)

Stacking Gel

For 1-4 gels:

2.5 mL Gel solution B
2 mL 40% acrylamide/1.2% Bis
0.2 mL 10% SDS
2 mL glycerol
13.3 mL deionized water

Degas under vacuum (do not allow to foam)
May use half volumes for 1 to 2 gels

Overlay Solution

2 mL Gel solution A
0.16 mL 10% SDS
14 mL deionized water

Degas under vacuum (do not allow to foam)

APPENDIX B

MISCELLANEOUS PROTOCOLS

Cresyl Violet Nissl Stain

Prepare Cresyl Violet solution 0.1% by weight Cresyl Violet in water with 3% glacial acetic acid. Filter prior to use.

Dip slides sequentially in:

1. Cresyl Violet solution: 2 minutes
2. Distilled water: 30 seconds
3. 50% EtOH: 30 seconds
4. 75% EtOH: 30 seconds
5. 100% EtOH: 30 seconds
6. Xylene: 2 minutes

Mount slides with Permount or Entellan™

Polyacrylamide Gel Electrophoresis

1. Assemble gel plates and prepare apparatus
2. Add ammonium persulfate to running gel (120 μL in 60 mL) and overlay solution (200 μL in 16 mL)
3. Pour running gel to height of 5.5 cm
4. Gently float 1 mL overlay solution on top of running gel
5. Allow to polymerize, pour off overlay, rinse gel top with water
6. Add ammonium persulfate to stacking gel solution (200 μL in 20 mL)
7. Pour stacking gel, insert comb
8. Allow to polymerize, remove comb
9. Install gels in tank, cover with tank buffer
10. Load samples
11. Run at 120V to 200V until blue dye just moves off bottom of gel (0.5 to 1 h)